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# MACROPHAGES, NUCLEIC ACIDS, AND THE INDUCTION OF ANTIBODY FORMATION*

## A REVIEW

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### INTRODUCTION

The problem of how antibodies are formed has plagued immunologists since the beginning of this century. Periodically, new ideas, or more often, modifications of older ideas, appear and serve to keep interest alive and to stimulate the development of new experimental methods.

Currently, impetus has been provided by a reexamination of Ehrlich's belief in 1900 that antibody is a normal product of body cells (Talmage, 1957); that is, that antibody consists of that portion of a broad spectrum of naturally occurring globulins for which a particular antigen has affinity. According to this concept antigen does not act as a template upon which new antibody is molded, either directly or indirectly, but rather it serves to stimulate the production of the natural globulin with which it is reactive.

Two different mechanisms have been proposed in recent years to explain such a process. Jerne (1955) suggested that combination of foreign protein occurs with natural globulin in the circulation and that the complex is then phagocytized. Ingestion of the antigen-bound globulin then leads to synthesis of more of the same kind of globulin. The antigen, having served merely to carry the globulin into the cell, is released into the circulation free to repeat the process. In this way selective synthesis of a particular species of globulin accounts for the observable antibody response.

Consideration of Jerne's theory of "natural selection" led other workers (Talmage, 1957 and 1959; and Burnet, 1957 and 1959) to suggest the whole cell as the unit of globulin production which is selectively stimulated by antigen. Accordingly there would exist from embryonic development for every conceivable antigen a cell line already producing globulin reactive with it. Introduction of antigen results in reaction between it and its complementary natural globulin in such a way (probably at the cell surface) as

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to stimulate proliferation of those cells producing the globulin. The accomplishment of this "preferential proliferation," to quote Burnet, results in detectable levels of serum antibody produced by the progeny of initially stimulated cells.

This latter concept which Burnet calls "clonal selection" has been favorably discussed by Lederberg (1959) in terms of genic control of globulin synthesis. Lederberg, drawing from experiences in genetic studies of microbial populations, suggests that the multiplicity of cell types required to account for globulin diversity is maintained by a high rate of spontaneous mutation in precursor cells throughout the lifetime of the organism.

There is thus far no experimental verification for this "elective" concept of antibody formation as Lederberg terms it. Experiments to determine the antibody potential of single cells are providing information of this sort (Nossal and Lederberg, 1958; Nossal, 1959; and Attardi, Kohn, Horibata and Lennox, 1959). Attardi and his coworkers succeeded in detecting individual cells producing two different antibodies; no triple producers were detected. Assuming only one active locus controlling cellular antibody potential, one would expect a maximum of two types of antibody to be produced by a diploid heterozygote; however, Lederberg points out that this "genotypic restriction" need not be evident if the cell's recent ancestors had undergone a series of mutations at the antibody locus. When tested such a cell might display several additional antibody characters due to hold over of phenotypic information. In such a situation, Lederberg observes, one would have to test the cells of clones derived from multipotential single cells in order to show dilution of the "phenotypic residue."

In addition both Lederberg (1959) and Burnet (1959) note that should the self-replicating unit of antibody production be subcellular, that is microsomal, the population of antibody forming units would be of such enormity as to make a cell's potential practically limitless.

Although "clonal selection" is highly challenging speculation, we wish to concern ourselves in this review with earlier proposals which we feel have not been so adequately tested as to justify their discard. As our title indicates we would refocus attention on the macrophage and its possible role in antibody formation. In doing so we will return to suggestions made by Burnet and Fenner (1949) and by Wissler, Fitch, LaVia and Gunderson (1957) concerning the mediation of an inductive substance between antigen ingesting cell and antibody producing cell.

Antibodies are proteins which appear to arise *de novo* from the cellular pool of free amino acids (Gros, Coursaget and Macheboeuf, 1952; Greene and Anker, 1954; Wolf and Stavitsky, 1956; and Stavitsky and Wolf, 1958).

The protein produced in antibody synthesis is of a distinct type, that is, in reacting with the appropriate antigen it displays a specificity not possessed by the remainder of the gamma globulins. This capacity of the antibody-forming cell or cells to produce alternative differentiated proteins has an analogy in the development from the fertilized ovum of a number of distinct cell lines each of which has a specificity presumably derived from its

structural elements. Indeed, Fraser (1959) has speculated that cytodifferentiation may result from an ability of cytoplasmic substances to act as inducers or repressers of protein synthesis in a manner analogous to the induction of antibody synthesis by antigen. Thus, as do others (Talmage, 1959; and Lederberg, 1959) we feel that information obtained from studies of cell growth and differentiation should be of value in the investigation of the antibody response, and pertinent aspects of this subject will be discussed here.

## MACROPHAGES

*Definition.* It has been demonstrated repeatedly that a particulate antigen injected into an animal is taken up immediately by phagocytic cells (Kyes, 1916; Motohashi, 1922; Philipson, 1936; Taliaferro and Taliaferro, 1955; and Fitch, Barker, Soules and Wissler, 1959). These cells are not confined to any one area of the body but are found both fixed and wandering throughout the tissues. The whole system of phagocytic cells was termed the reticulo-endothelial system (R.E.S.) by Aschoff and Landau in 1913 (Aschoff, 1924). The distinctive feature of phagocytes according to Aschoff is their ability to ingest both particulate and solubilized dyestuffs. The terms macrophage and monocyte are frequently used to designate the cells of the R.E.S. obtained from various sources. Carrel and Ebeling (1926) felt these cells represent a single type and need not be distinguished. They concluded from comparative tissue culture that both the macrophage and the monocyte assume the same form if cultured under identical conditions. Maximow and Bloom (1957), however, draw a distinction between macrophage and monocyte, the monocyte being unable to store vital dyes. These authors, nevertheless, agree that the monocyte can develop into a macrophage. Carrel and Ebeling (1926) also listed a number of other synonyms often encountered in the literature—polyblast, clasmatocyte, endothelial leucocyte, adventitial cell, large mononuclear leucocyte, and blood histiocyte. The term macrophage will be used exclusively throughout this report.

*Accumulation of antigen and antibody formation.* The finding of antigenic material in macrophages originally led to the belief that the R.E.S. was the site of antibody formation. A number of studies were carried out attempting to justify this concept. These involved interference with R.E.S. activity and attempts to correlate such interference with a concomitant decrease in antibody formed following an antigenic stimulus. Interference with R.E.S. function was accomplished either by "blockade" with dyes, India ink, or iron particles or by actual removal of various organs rich in phagocytic cells. The results of these experiments varied considerably. A number of investigators found that interference with normal R.E.S. function either significantly diminished or completely inhibited antibody production (Bieling and Isaac, 1922; Motohashi, 1922; Siegmund, 1922; Gay and Clark, 1924; Portis, 1924; Jungeblut and Berlot, 1926; Cannon, Baer, Sullivan and Webster, 1929; and Tuft, 1934). Others indicated that no such diminution of the antibody response followed R.E.S. "blockade" (Rosenthal and Fischer,

1922; Standenath, 1923; and Ross, 1926). Standenath actually showed accelerated precipitin development to horse serum in a rabbit which had been "blockaded" by India ink treatment. By 1931 in a review of the reticuloendothelial system in immunity Jaffe was able to cite a large number of investigations of this type the results of which were equally divided between depression, lack of effect, or stimulation of antibody formation.

The conflicting reports from "blockade" experiments can best be attributed to the failure of the investigators to standardize the conditions of "blockade." Those who obtained the greatest correlation between inhibition of antibody synthesis and R.E.S. interference appear to have achieved a much higher level of "blockade." Benacerraf (1960) has pointed out that it is important to inject enough particles to challenge all of the phagocytes. It can be concluded that interference with normal macrophage activity with respect to the accumulation of antigen does indeed depress the antibody response although, as will be pointed out, this depression does not result from a direct effect on the antibody forming cell.

Studies in which comparative antibody titrations were performed on serum and on macrophage-rich tissues from immunized animals indicated that the tissue antibody frequently appeared earlier or in higher titer. The comparative titers in these macrophage-rich areas (induced by repeated inoculation of antigen or inert particulates) were taken as circumstantial evidence for macrophage participation in antibody formation (Cary, 1922; Cannon and Sullivan, 1932; Walsh and Cannon, 1934; and Hartley, 1940).

In 1939 Florence Sabin employed dye-linked egg albumin as antigen and showed conclusively that the route of inoculation into a rabbit determined the site of antigen ingestion. Intravenous injection resulted in phagocytosis of the antigen by macrophages of the splenic pulp, liver (Kupffer cells), and bone marrow. Intraperitoneal injection resulted in antigen accumulation by macrophages of the omentum, peritoneum, and retrosternal lymph nodes, while intradermal or subcutaneous inoculation led to the amassment of antigen by macrophages in the regional lymph nodes. Sabin also observed macrophages shedding portions of their "surface film" when the living cells were examined under the microscope. Shedding was enhanced following the administration of antigen. She correlated the appearance of antibodies in the serum with the shedding process and the disappearance of antigen. Sabin postulated that antigen is ingested into vacuoles of the macrophage where it is solubilized and then passed into the cytoplasm. The mere presence of the altered antigen in the cytoplasm activates in some way the production of both normal and antibody gamma globulins which are then passed into the plasma.

Although the cited studies support the hypothesis that macrophages synthesize antibodies, none are definitive. Sabin did not actually demonstrate antibodies in the shed cytoplasm. The extraction of antibodies from tissues containing large numbers of macrophages was no more than presumptive evidence (which the more direct technique of fluorescent antibody staining has failed to confirm (Coons, Leduc and Connolly, 1955)) that such cells were

the actual sites of antibody formation. Moreover the liver, which contains a very large population of macrophages, while producing other plasma proteins does not appear to produce gamma globulin (Miller and Bale, 1954; and Askonas, Humphrey and Porter, 1956). These studies noted the specific failure of perfused whole rat livers or rabbit liver slices to incorporate $C^{14}$-labeled amino acids into gamma globulin.

It must be noted that although Ranney and London (1951) reported the synthesis of small amounts of antibody by rat liver slices *in vitro*, their product could be accounted for by the presence of contaminating plasma cells (Coons, Leduc and Connolly, 1955; and Thorbecke and Keuning, 1956) which must be taken into account when considering earlier studies of macrophage participation in antibody formation. Indeed, the theory that macrophages produce antibody appears unacceptable at present.

## LYMPHOCYTES AND PLASMA CELLS

Other workers have obtained similarly indirect evidence indicating that lymphocytes and plasma cells are involved in antibody formation. Antibodies are found in lymph nodes draining the sites of intracutaneously or subcutaneously injected antigens (McMaster and Hudack, 1935; and Oakley, Warrack and Batty, 1949) and are in higher titer in the efferent than in the afferent lymph (Ehrich and Harris, 1942).

Disintegration of lymphocytes in blood and tissue brought about by injecting pituitary adrenotrophic hormone or adrenal cortical extract was reported to result in a simultaneous rise in antibody titer in immunized rabbits and mice (Dougherty, Chase and White, 1945). Similar increases in circulating antibody are not noted, however, following lymphocyte destruction by x-rays or nitrogen mustard (Marshall and White, 1950).

Ehrich and Harris (1942) noted that along with the detection of antibody in the efferent lymph from popliteal lymph nodes, there was a marked proliferation of lymphocytes within the node. Others, however, have found increases in plasma cells in both lymph nodes and spleen associated with the appearance of circulating antibody (Thorbecke and Keuning, 1953; and Roberts and Dixon, 1956).

Fagraeus (1948) removed splenic red pulp rich in plasma cells from rabbits during the anamnestic response and obtained the formation of antibody *in vitro*. Explants of lymph follicles with a high content of lymphocytes rather than plasma cells failed to yield antibody. Fagraeus attributed antibody production to immature rather than mature plasma cells. Experiments of a like kind were conducted by Keuning and van der Slikke (1950) who agreed with Fagraeus's conclusions.

Antibodies have been found in extracts of tissues rich in plasma cells (Bjorneboe, Gormsen and Lundquist, 1947) and have been demonstrated immunohistochemically by Coons and his coworkers (1953) using the fluorescent antibody technique. The finding of antibody within a cell need not indicate that it was produced by that cell (Wissler, Fitch, LaVia and Gunderson, 1957) and is therefore no more conclusive than studies of mixed-cell populations.

Perhaps the most elegant studies of this problem have been those involving the production of antibodies by single cells (Nossal, 1959; and Attardi, Cohn, Horibata and Lennox, 1959). In each of these investigations individual cells were isolated from the lymph nodes of immunized animals, placed in microculture for a few hours, and then assayed for antibody.

Nossal's system employed bacterial flagella as antigens. The resultant antibody was titrated by its immobilization of the specific bacteria introduced into a microdroplet containing a single cell.

Attardi and his associates used bacteriophage antigens. They incubated isolated cells with the phage in microdroplets and then plated the mixture on an indicator strain of bacteria. The amount of antibody was determined by the relative amount of virus neutralized.

As a result of these studies it appears that only a small percentage (ten to 20 per cent) of the cells in a lymph node produce antibody to a particular antigen. Of the cells which were found to produce antibody the greater proportion were plasma cells. It is on this point that the results of these two experiments differ. According to Nossal virtually no lymphocytes produced antibody (one out of 306 tested) while Attardi's group found more than ten per cent of the lymphocytes tested were producing antibody.

This difference cannot readily be explained. These studies differed as to antigen, course of immunization, test animal employed, and method of antibody titration. It is conceivable that any of these dissimilarities could account for the discrepancy. It is additionally possible that there is disagreement as to what constitutes a lymphocyte.

Nossal also reported testing a very small number of macrophages (23), none of which made detectable antibody. Thus, although establishing the plasma cell as the major producer of antibody, these experiments have left uncertain the role of the other cell types.

## INTERRELATIONSHIP OF CELL TYPES

The relationships, if any, between plasma cells, lymphocytes, and macrophages are unclear. Fagraeus (1948) felt that the mature plasma cell represented the final stage in a chain of development originating with a reticulum cell of the R.E.S. Marshall and White (1950) described a primitive reticulum cell which was not phagocytic, but which could develop into either a plasma cell or a lymphocyte. Their conclusion was drawn from histological examination of tissues at various intervals after antigen administration. It seems the reticulum cell described by Fagraeus could not have been of a type identical to that of Marshall and White since the cell she described was a part of the R.E.S. and hence, by definition, phagocytic. It should be pointed out that Marshall and White were unable to find evidence for a transition from reticulo-endothelial cell to plasma cell as Fagraeus had claimed.

Other investigators have felt that both the macrophage and/or the lymphocyte may give rise to plasma cells (Roberts and Dixon, 1956; Roberts, Dixon and Weigle, 1957; and Nossal, 1959). On this basis Dixon, Weigle and Roberts (1957) conclude that lymph node lymphocytes and the macrophages

of peritoneal exudates are primarily responsible for antibody formation. Wissler, Fitch, LaVia and Gunderson (1957) have summarized studies involving the intravenous injection of mixtures of India ink particles and $I^{131}$-labeled typhoid bacilli. They found carbon particles and $I^{131}$ activity localized in the same phagocytic cells of the spleen, but the carbon particles were not found in the proliferating splenic red pulp cells associated with antibody production. Their data indicate that the antibody-forming cell is derived from non-phagocytic cells. These workers further state that the ability of the circulating lymphocyte to develop into a macrophage is "well-established"; that is, they agree with those hematologists who have observed transitional forms in the series, lymphocyte to monocyte to macrophage (Maximow and Bloom, 1957). They (Wissler, Fitch, LaVia and Gunderson) conclude from histologic studies of immunized rat spleens that the antibody synthesizing cell "appears to be a large, pyroninophilic cell derived by active mitosis from a primitive fixed reticular cell." This cell which does not appear to be phagocytic can develop into either a small lymphocyte or a mature plasma cell after producing antibody to a primary antigenic stimulus. Sterzl (1959) also suggests this type of sequence but does not clearly define the status of the antigen which must come in contact with the primitive cell.

## MODIFICATION OF ANTIGEN

Wissler and his coworkers (1957) in accord with Burnet and Fenner (1949), propose a mechanism of antibody formation in the rat spleen whereby antigen is phagocytized by the macrophage and in an unknown manner acted upon and released in the form of a "modified antigen." The "modified antigen" can in turn act on the primitive reticular cell transforming it into an antibody producing cell.

There is some justification in the literature for this proposal. Garvey and Campbell (1956 and 1957) have reported the localization and persistence of $S^{35}$-labeled antigens in rabbit liver. These labeled proteins in fragmented form appeared associated with ribonucleoprotein; that is, with a salt soluble nucleic acid-like material with an absorption maximum of 2575 angstroms. The ribonucleoprotein-complexed antigen when isolated from the liver was more highly antigenic in that less of this material than of whole antigen was required to sensitize a guinea pig. As mentioned earlier, the liver does not synthesize gamma globulin. It would seem then that if the antigen found in the liver is to have a role in antibody formation, it or information from it must somehow be transferred to sites of antibody synthesis.

Stevens (1959) studied the immune response of wax moth larvae to *Pseudomonas aeruginosa* antigen. She observed that the antigen could be found in the blood of the insect in an altered form. The injection into guinea pigs of such antigen recovered from insects produced an agglutinating titer ten times higher than did corresponding amounts of normal antigen. Stevens suggested the possibility that the antigen was ingested by insect cells and secreted in an altered, more antigenic form.

Dixon, Weigle and Roberts (1957) found that irradiation of macrophages *in vitro* interferred with the ability of the cells to transfer a secondary antibody response to ordinarily responsive normal recipient rabbits. They decided that macrophages possess the "immunologic functions" necessary for the transfer of antibody synthesis. Fishman (1959) reported the production of antibodies by rat lymph node cells *in vitro*. Fishman's system required preincubation of the antigen with macrophages which were then ground and extracted. The extract was then added to the lymph node cells. Peak antibody titers were obtained after 11 days incubation of the mixture. Fishman noted that carefully prepared homogenates of macrophages could also be used to pretreat the antigen and also that the macrophages had to be from the same species of animal as the lymph node cells.

Other workers (Sterzl and Hrubesova, 1956) have reported the successful induction of antibody formation in non-immunized baby rabbits with ribonucleoprotein from the spleens of immunized adult rabbits. Antigen could not be detected in the ribonucleoprotein preparations. Control baby rabbits did not respond to injections of whole antigen. (However, see Hrubesova, Askonas and Humphrey, 1959.)

## NUCLEIC ACIDS AND SUBCELLULAR PARTICLES

The apparent association of antigen with ribonucleoprotein is of considerable interest in light of current concepts of protein synthesis. Amino acids bound to soluble RNA are believed to be transferred to the microsomes (Hoagland, Stephenson, Scott, Hecht and Zamecnik, 1958) where the amino acid sequence of the protein is determined (Schweet, Lamfrom and Allen, 1958). Zamecnik and Keller (1954) had demonstrated earlier that radioactively labeled amino acids were incorporated into the proteins of a microsomal fraction in what appeared to be true peptide linkage.

Specific proteins (serum albumin, Campbell, Greengard and Kernot, 1958; and Peters, 1959; antibody, Askonas, 1958; and Kern, Helmreich and Eisen, 1959) have been found in or on the microsome suggesting synthesis at this site.

The microsomes to which amino acids are transferred are small basophilic granules (rich in RNA) in the cytoplasm (Palade, 1955a). The microsomal granules line the outside of the membranes of the endoplasmic reticulum, a series of membrane-bound cavities throughout the interior of the cell (Palay and Palade, 1955).

Although the association of microsomes with protein synthesis appears best defined, it should be noted that the nucleus (Allfrey, Mirsky and Osawa, 1957) and mitochondria (Bates, Craddock and Simpson, 1958; and Kalf, Bates and Simpson, 1959) also synthesize protein.

A number of workers have attempted to show that injected antigens localize in or on particular cell structures. Haurowitz, Crampton and Sowinski (1951) traced antigens first to the microsomes (corresponds to the endoplasmic reticulum in current terminology) and then to the mitochondria and nuclei of liver cells. Coons (1956) has also reported detecting antigen in

the nuclei not only of liver cells but of others as well, such as adrenal cortical and renal tubular cells. Antigen was also found in liver mitochondria by Fields and Libby (1952) and Erickson, Hensley, Fields and Libby (1957). Hawkins and Haurowitz (1959) detected intravenously injected $S^{35}$-labeled proteins in rat spleens. Using specific antisera to precipitate antigen in splenic extracts they found the protein associated mainly with ribonucleoprotein; however, a very small fraction appeared associated with deoxyribonucleoprotein, suggesting a nuclear location for some of the material.

Whether an antigen is in the nucleus or the cytoplasm of a cell may not be of particular importance in view of recent evidence concerning the nature of the nuclear membrane. It appears that the nuclear membrane is actually continuous with the endoplasmic reticulum (Afzelius, 1955; Watson, 1955; De Groodt, Derom, Lagasse, Sebruyns and Thiery, 1958; and Yasuzumi, 1959). Previously Palade (1955b) had shown that invaginations of the plasma membrane frequently ran deep into the interior of the cell body and that these infoldings connected with the interior of the endoplasmic reticulum. If these observations are correct, they indicate how communication might be established between the nucleus and the outside of the cell (Schultz, 1959). Palade (1956) has proposed that flow along the surface of the plasma membrane may be important in the active transport of substances in both directions through the cytoplasm. Thus any single observation of an antigen within a cell may simply locate it at one point on the course it is taking. In spite of observations of an intimate association between protein forming structures and antigen no one has answered the question concerning the nature of the relationship.

In view of the long-lasting nature of the ability to respond to an antigen it has been suggested that antigen has as its primary role the modification of deoxyribonucleic acid (DNA) (Schweet and Owen, 1957; and Coons, 1958). Novelli and Demoss (1957) feel that RNA in the form of ribonucleoprotein (RNP) not only acts as an organizer of proteins by providing information governing the sequence of amino acids but also provides a structure on which polymerization of the amino acids can take place. It follows that a template for the formation of antibody could be RNP into which antigenic groups have been incorporated or RNP which has been modified by antigenic groups. It is apparent that if RNP ordinarily organizes protein synthesis it would also be involved in any theory which views antibodies as normal cell products and antigenic modification would not be required.

Although a template for protein synthesis has not been experimentally defined, the concept appears well established. Dalgliesh (1958) and Koshland (1958) in recent articles indicate that the template concept is chemically feasible and that a sequential process of amino acid incorporation into protein appears likely. Such a stepwise linkage of amino acids on a template is supported by experimental data obtained by Loftfield and Eigner (1958) who measured the rate of incorporation of labeled amino acids into rat liver ferritin. Moreover, Rabinovitz and Olson (1959) interpret results obtained by blocking hemoglobin synthesis with an amino acid analogue to

indicate a failure of the synthesized protein to assume a proper configuration. They postulate accumulation of protein intermediates. Such intermediates would be expected to accumulate according to Koshland (1958) since protein would not be released from the template until the whole molecule had been synthesized. Askonas and Humphrey (1958) found that in hyperimmunized rabbits antibody appeared to be first synthesized in a bound form.

## RADIOMIMETIC AGENTS AND RADIATION

There are few data on which to base a choice between DNA or RNA mediation of antibody formation. Schwartz, Eisner and Dameshek (1959) found that 6-mercaptopurine, a purine analogue, completely blocked the primary response to a soluble protein antigen while the same dose of analogue only partially decreased a secondary response. Dutton, Dutton and Vaughan (1959) reported that 5-bromodeoxyuridine, a specific antagonist of thymidine (DNA precursor), prevents the formation of antibody protein by rabbit spleen *in vitro*. The inhibition of antibody synthesis was overcome in the presence of excess thymidine. The addition of excess uridine (RNA precursor) had no effect.

Depending on the dose and time of irradiation in relation to antigen administration the antibody response can be prevented or delayed (Taliaferro, 1957) by a mechanism not yet known. Nonspecifically, carbon particles (Strauch, Stender and Winter, 1959) and bacterial endotoxin (Kind and Johnson, 1959) have been shown to alleviate partially the effects of x-ray on antibody formation.

Jaroslow and Taliaferro (1958) reported that hemolysin production was normal in highly x-irradiated animals if they were given an enzymatic digest of nucleic acids at the time of antigen injection. Highly polymerized nucleic acids had no effect. These results suggested to them that the nucleic acid digests contained substances normally required for mediation of the interaction between nucleotide and antigen. Kinetin, a purine derivative of DNA known to increase mitosis many fold (Paschkis, 1958), was found to be as active in restoring antibody synthesis as the nucleic acid digests.

Such data are suggestive of DNA-nuclear participation in antibody formation perhaps by inciting proliferation of antibody producing cells. The latter suggestion might be drawn from a report by Firshein and Braun (1958) summarizing the ability of DNA digests to induce shifts in bacterial populations. They indicate that the digests, as well as kinetin, may function by either a selective inhibition or stimulation of specific cell types which is probably associated with an altered rate of DNA synthesis. Firshein and Braun note that the phenomenon might be widespread as indicated by the effects of the digests not only on many types of bacteria but also on mouse lymphosarcoma cells and on the antibody forming system as noted in the Jaroslow and Taliaferro (1958) experiments.

Radiation's effect on antibody formation might be explained by the elimination of antibody forming cells either by direct destruction, an explanation

favored by Burnet (1959), or indirectly through prevention of cell division by interference with nucleic acid synthesis (Nygaard, 1959; Van Lancker, 1959; and Harbers and Heidelberger, 1959). Such an explanation well fits the clonal selection theory which requires cellular proliferation for antibody expression. Alternatively, assuming antibody is the end result of nucleoprotein transferred antigenic intermediates, it is possible that the destructive effect of radiation could result in nucleic acid disorganization out of which might arise aberrant intermediates capable of participating in cellular metabolism. This would appear possible since, to quote Puck (1960), "irradiated cells which have lost the power of sustained reproduction can carry out active metabolism including many kinds of specific macromolecular biosynthesis for days or weeks." Under these conditions x-ray-modified nucleic acid, especially RNA, could then directly affect antibody synthesis in terms of the final product.

### CONCEPT OF "ABERRANCY" IN PROTEIN SYNTHESIS

Doudney and Haas (1959) studied the effects of ultraviolet light-(UV)induced mutation to the non-tryptophan-requiring state in *Escherichia coli.* They found that chloramphenicol added to irradiated cells held in an amino acid-rich medium prior to plating resulted in a decreased mutation frequency. The ability of "chloramphenicol challenge" to give this result depended on the time of addition. After 75 minutes in the holding medium cells were no longer subject to "mutation frequency decline" if chloramphenicol were added at this point. The mutation had been fixed. Doudney and Haas noted that RNA synthesis paralleled mutation fixation whereas DNA and protein synthesis occurred later. They therefore proposed "the principal mutagenic effect of UV is to modify ribonucleic acid precursors present in the cell at the time of radiation exposure."

Doudney and Haas further found that 5-hydroxyuridine, a uridine analogue, causes a decrease in the number of mutations occurring in a given population of *E. coli* following UV irradiation. They correlated this decline in mutation frequency with RNA synthesis proposing that the 5-hydroxyuridine was incorporated into RNA with the resultant production of "nonfunctional" RNA. Hosoda, Kohiyama and Nomura (1959) found that 8-azaguanine, a guanine analogue, blocked the synthesis of amylase by a uracil requiring mutant of *Bacillus subtilis* only in the presence of uracil. This suggested to Hosoda's group that the analogue acted by being incorporated into an "unphysiological nucleic acid" and thus that the analogue could block enzyme synthesis only by participating in nucleic acid synthesis.

Not all abnormal RNA need be biologically inactive. The ability of altered RNA to lead to the production of aberrant proteins has been proposed and products of this type have reportedly been obtained (Matthews, 1957; Chantrenne, 1959; Jeener, 1959; and Hamers and Hamers-Casterman, 1959).

Although UV and x-ray may have different primary effects (Jacob, 1954), it is nevertheless possible that in the x-ray-antibody phenomenon there is operative a mechanism similar to that proposed by Doudney and Haas (1959)

in which RNA precursors are altered by UV irradiation prior to their incorporation into RNA. If similarly modified RNA occurs in mammalian cells, then radiation may not actually block the formation of immune bodies but rather may affect the specificity of the protein being synthesized. It is proposed that the antigen still stimulates the production of antibodies, but that these are not recognizable as such because the globulin produced is an aberrant form resulting from the metabolism of abnormal RNA.

In line with such reasoning we (Rittenberg and Nelson, 1960) reported the effects of immunization on the globulin levels of irradiated rabbits. We noted that a single injection of bovine serum albumin into rabbits which had received 400 roentgens whole-body irradiation 24 hours previously resulted in increased levels of gamma and beta globulins. The increases in globulin were the same in both the irradiated and unirradiated immunized animals; however, only the unirradiated rabbits produced antibodies detectable by immune elimination of radioactive antigen or by ammonium sulfate precipitation of iodine$^{131}$-labeled antigen-antibody complexes. While this preliminary experiment does not constitute proof of the modified RNA concept, it is difficult to explain by cell destruction since unimmunized, but irradiated controls did not show similar increases. Neither do these results easily fit a concept of antigenically induced selective cellular proliferation as the basis of antibody formation since here antigen would have had to select other than the appropriate cell line in order to account for the globulin increase.

## NUCLEIC ACID AND INDUCTION OF DIFFERENTIATION

That antigen ingested by one cell may be released in modified form to initiate antibody synthesis in a second cell is worthy of further investigation. Burnet (1956) proposed that the template of the protein-synthesizing mechanism from macrophages, "with or without other components," might induce antibody formation in adjacent cells. Antigen modified ribonucleoprotein could qualify as mediator between cells. The studies already cited concerning the association of antigen with RNA and the transfer of antibody forming capacity by ribonucleoprotein lend weight to this assumption (Garvey and Campbell, 1956, 1957; Sterzl and Hrubesova, 1956; and Hrubesova, Askonas and Humphrey, 1959). The RNA of several animal viruses is infectious in the absence of viral protein (Colter, Bird and Brown, 1957; Alexander, Koch, Mountain, Sprunt and Damme, 1958; Brown and Stewart, 1959; and Schaffer and Mattern, 1959). Furthermore, Holland, McLaren and Syverton (1959), using viral RNA as the infective agent, were able to extend the host range of several enteroviruses to normally immune non-primate cells. Their experiments suggest that RNA may be capable of directing biosynthetic activities in a wide range of cells provided the RNA can gain entry in an active form.

Hayashi (1958) reviewed evidence that guinea pig liver ribonucleoprotein could induce the formation of neural tissue from undifferentiated *Triturus* ectoderm and felt that the inductive ability resided in the protein moiety of RNP. Niu (1958), however, in discussing Hayashi's experiments indicated

that RNA itself had not clearly been ruled out as inducer, and he added evidence of his own that similar inductive activity in calf thymus RNP extracts was more likely associated with RNA than with protein. Niu proposed that the protein of RNP might serve to stabilize or carry RNA or facilitate its entrance into cells. The protein might also serve to determine which cell can be penetrated by the RNA. The data of Holland and his coworkers (1959) indicated that the viral protein conferred host range specificity on viral RNA which once deproteinized could infect a wide variety of cells. Brachet (1957) notes that either RNA or protein could be important in induction depending on the species but that experiments of this nature must be carefully controlled to rule out the possibility of non-specific induction by toxic substances.

Since certain viruses, bacteriophage and transforming principles, are essentially DNA, this cannot be overlooked as a possible direct inducer in antibody formation.

## REEXAMINATION OF THE ROLE OF THE MACROPHAGE

The macrophage, the cell that takes up antigen, is the logical source of an "inducer" as suggested by Wissler and his coworkers (1957). One of the natural functions of macrophages is recognizing foreign and denatured materials. The average life span of a human erythrocyte is approximately 120 days according to Maximow and Bloom (1957) who also note that erythrocytes are normally destroyed by liver and spleen macrophages. Apparently only those old cells which are no longer able to carry out their normal function are phagocytized. Macrophages must then "recognize" this distinction between young and old, normal and abnormal cells. Brandt, Bass, Dodd and Wright (1952) noted that treatment of rabbit erythrocytes with specific immune sera enhanced their phagocytosis by rabbit spleen macrophages. They found that trypsinization of rabbit or human erythrocytes had a similar effect, normal human erythrocytes not being phagocytized at all. Claude, Dodd, Brandt, Elliot and Bass (1953) further noted that normal rabbit macrophages could distinguish between erythrocytes from normal and diseased humans since only those from diseased individuals were phagocytized. Because "recognition" is of such fundamental importance in determining to which substances antibodies are made, it follows that cells normally versed in this function should be best suited to identifying antigens.

According to Taliaferro and Taliaferro (1955) the macrophages of the spleen are more active in phagocytosis than those of the liver, but because of its size the liver is more important in ingesting antigen. Even though the liver takes up more antigen than any other organ it does not produce gamma globulin. Any antigen localized in the liver may therefore be lost as a stimulus for antibody production unless it is possible that the liver serves as a source of "inducer" following the intracellular interaction of antigen with nucleic acid material.

The liver, rich in macrophages, is known to have growth stimulating properties. Partial hepatectomy in one member of a parabiotic pair results in

increased mitosis and DNA synthesis in the nonhepatectomized member (Van Lancker and Sempous, 1959). Paschkis (1958) summarized data indicating that liver extracts can stimulate the growth of liver tumors and of other tissues. The growth promoting factor is not tissue specific. Kelly and Jones (1953) suggest that the active growth promoting fraction of liver is a deoxyribonucleoprotein. Their fraction also had stimulatory effects on the spleen. It is interesting to note that severe liver disease may result in increased levels of antibody (Havens, Shaffer and Hopke, 1951).

Wissler, Fitch, LaVia and Gunderson (1957) in the cellular concept of antibody formation described above suggest that the antibody forming cell once differentiated by contact with "inducer" gains a capacity to react directly with antigen by proliferating and producing more antibody. In such a system one might divide the anamnestic response into two separate reactions. One would consist of a true secondary response by predifferentiated cells. The second would correspond to a new primary response in which antigen is again taken up by macrophages, "modified," and "inducer" released to stimulate primitive reticular cells to produce primary antibody. According to this concept one would expect that the larger the primary dose of antigen (up to a saturation level) the more cells would be primed for the secondary response. As we have proposed, x-radiation modifies nucleic acid during synthesis and prior to its becoming an inducer in the primary response. Assuming that cells which have already been differentiated by a previous antigenic stimulus do not require contact with "inducer" in order to react directly with antigen, antibody production by these cells would be radioresistant. The more cells primed for the secondary response, the less radiosensitive would this response appear. Such results were reported by Taliaferro and Taliaferro as cited and confirmed by Talmage, Freter and Thompson (1956). They found that with larger doses of sheep erythrocytes as both primary and secondary stimuli the anamnestic hemolysin response was less sensitive to irradiation. The results obtained by Schwartz, Eisner and Dameshek (1959) with 6-mercaptopurine might also be explained in this way. They found less inhibition of the secondary than of the primary response.

The preceding discussion evinces our acceptance of the concept that the antibody response is not a unique biological event. We see in the development of living things a likeness to the process which results in the detectable change in serum indicative of antibody production. Noting that the antigen ingesting cell does not appear to produce antibody, we have discussed data concerning certain cell substances which have a capacity to induce phenotypic changes in other cells and have invoked such a mechanism to explain the action of macrophages (which ingest antigen) on antibody producing cells. Evidence linking nucleoprotein to protein synthesis and to cellular differentiation suggests that nucleoprotein serves as the intercellular mediator of antibody formation. More definitive evidence of the association of antigen with nucleoprotein and the heightened antigenicity of these complexes would further strengthen this idea. This review has raised ques-

tions concerning the "real" effect of radiation on the immune response and the initial role of antigen in stimulating primary antibody synthesis. Investigations focused on the macrophage and its nucleic acids will, we feel, aid in the solution of these problems.

## SUMMARY

Literature concerning antibody formation, protein synthesis, and cytodifferentiation is discussed with a view to relating the three phenomena. The data stressed suggest that antigen is ingested by macrophages of the reticulo-endothelium and "altered" in such a manner that antigenic information is contained in nucleoproteins. Nucleoproteins so informed are then able to induce a susceptible cell to produce antibody. The suggestion is made that modification of nucleic acid precursors by irradiation prior to immunization ultimately results in the production of aberrant forms of antibody protein. The liver, which appears to have no direct role in the immune response, is proposed as a logical organ with which to investigate the relationship between macrophage, nucleic acid, and the induction of antibody formation in other tissues.

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# EXPERIMENTAL STUDIES OF MIMICRY

## 5. THE REACTIONS OF TOADS (*BUFO TERRESTRIS*) TO BUMBLEBEES (*BOMBUS AMERICANORUM*) AND THEIR ROBBERFLY MIMICS (*MALLOPHORA BOMBOIDES*), WITH A DISCUSSION OF AGGRESSIVE MIMICRY

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### INTRODUCTION

The similar appearance of some flies and bees has been known since the time of Aristotle and the confusion of one with the other served as the basis of the ancient and false notion that bees spontaneously generated from decaying carcasses (Mackail, 1950). The idea that certain flies gain an advantage by looking like bees was first put forward by Kirby and Spence (1817), and later noted by Wallace (1871). These authors thought that the resemblance of Syrphid flies of the genus Volucella to bumblebees (Bombus spp.) enabled the flies to enter the bees' nests to lay their eggs without being attacked. When classifying the various categories of mimicry, Poulton (1890) placed this as an example of *aggressive mimicry*, a view which he maintained in several subsequent papers (1892a, 1892b). This is the situation in which one species resembles another unrelated one in order to approach it the better without exciting suspicion for various detrimental purposes. Later, however, Poulton was criticized by Bateson (1892) for supporting the idea of aggressive mimicry with the Volucella example, and in 1904, rescinded this interpretation calling it instead an instance of protective (Batesian) mimicry; that is to say, by looking like bees, the palatable flies gain protection from vertebrate predators which have learned that bees are noxious. His main reasons for abandoning the idea were that the Volucella females were observed to enter nests of Bombus which they did not mimic, and also because Fabre (1903) believed that a European species with similar habits was actually beneficial to its host, the fly larvae acting as scavengers inside the nest. It is, however, important to note that the role of Volucella larvae inside the bees' nests is by no means certain (Free and Butler, 1959), and the majority of Syrphid fly larvae are in fact predaceous (Clausen, 1940; Sweetman, 1958).

In the same paper, Poulton also discussed flies of the family Asilidae which in several instances prey as adults upon aculeate Hymenoptera to which they bear a highly specific and remarkable resemblance. Although considering the possibility of aggressive mimicry, he rejected it in favor of protective mimicry and this interpretation has been accepted until this time (Carpenter and Ford, 1933; Cott, 1940; Imms, 1951; Free and Butler, *loc.*

*cit.*; Linsley, in press). Nevertheless, Poulton's change in emphasis on the selective agents controlling the evolution of bee mimicry did not lessen his interest in the subject, and in 1906 he wrote an extensive review on predaceous insects and their prey in which he classified protective mimicry in the Asilidae into three groups ranging from those flies which mimic Hymenoptera but do not especially attack their models to those in which they prey exclusively upon them.

In 1924, Poulton published Van Someren's observations on asilids of the genus Hyperechia which mimic Xylocopid bees in South Africa. This evidence strongly suggested that the larvae of the asilids feed upon the larvae of their bee models, and two years later other investigators in southern India (Poulton, 1927a) and eastern Africa (Poulton, 1927b) showed that this is so in several other species of Hyperechia. Thus, in the Asilidae there are clear instances of mimicry in which the specific aculeate models are exploited in all stages of their life history by their mimics. It is notable that Poulton did not revive his earlier idea of aggressive mimicry which he had discarded partly because of the lack of evidence that aggression existed in the Volucella example. It is even more striking that this should be so in view of his knowledge of Cuckoo egg mimicry which he interpreted as similar to Batesian mimicry in the insects (1926). Moreover, later authors including Cott *loc. cit.* and Southern (1954) also mentioned several points of similarity between the two, and it is highly interesting that there is a group of Hymenoptera known as Cuckoo bumblebees (Psithyrus) whose behavior is analogous to that of the Cuckoo birds in that they exploit other bumblebees to which they bear a specific resemblance (Free and Butler, *loc. cit.*).

In our opinion the birds are a clear example of aggressive mimicry, not only in that their eggs resemble those of the foster parents, but also in some cases the nestling Cuckoos themselves resemble the hosts' young (Jourdain, 1925). Thus, it appears that the phenomenon of aggressive mimicry does exist in the animal kingdom, and this may now be defined as follows: aggressive mimicry is the superficial visual similarity of some stage in the life history of a predator to its prey or a parasite to its host; as such the predator or parasite is the aggressive mimic and the prey or host the model. The mimicry facilitates the mimic's exploitation of its model and has evolved because of the visual selection exerted through the defensive behavior of the model against those forms of the mimic which least resemble it.

A possible objection to this idea in the insects is that the sense of sight in the Hymenoptera may not be well enough developed to account for the high degree of resemblance between the model and mimic. From what is known of the morphology and physiology of the compound eye, its visual acuity seems far below that of the vertebrate camera eye. Nevertheless, both field observations and conditioning experiments with aculeates have indicated that their pattern discrimination must be fairly efficient (Dethier, 1953; Carthy, 1958). For example, Tinbergen (1935) experimentally demon-

strated that the hunting wasp, *Philanthus triangulum* Fabricius, which preys upon honeybees, recognizes them from a distance by sight alone. Moreover, the phenomenon of mimicry in orchids in which male bees are attracted to and copulate with the flowers that specifically resemble their females is another strong argument favoring well developed visual discrimination in bees (Sheppard, 1958).

From these considerations, it seems possible that the mimicry of aculeate Hymenoptera by flies or by other bees could be advantageous both for aggressive purposes of one towards the other, and for protective value with regard to their vertebrate predators. Thus the fly mimicry could be aggressive and Batesian and the Cuckoo bee mimicry aggressive and Müllerian. In the latter form of mimicry, by resembling each other, fewer individuals of both bee species would be killed in the education of their vertebrate predators.

The purpose of this paper is twofold: first, to present the results of a field study made in south central Florida on the aggressive behavior of an asilid fly mimic towards its model bumblebee, and secondly, to describe a series of laboratory experiments which were designed to test the hypothesis of Batesian mimicry in this instance.

## THE FIELD STUDY

Several different genera of the Asilidae occur in the southeastern United States and among them is the genus Mallophora which includes five species that mimic bumblebee workers or queens (Bromley, 1925, 1950). The genus is well known because the various species have been reported as economic pests of honeybees (*Apis mellifica* Linné). The species upon which our work was done is *Mallophora bomboides* Weidemann, called the Florida Bee Killer. In addition to preying upon honeybees, it has also been reported eating its model, *Bombus americanorum* Fabricius workers, carpenter bees, wasps, and when Hymenoptera are scarce, large beetles, reduviid bugs, and grasshoppers (Bromley, 1930). Its distribution is from Florida where it occurs in the dry sand scrub, north to Wilmington, North Carolina, along the sandy coastal strip. In the same paper, Bromley also noted the loud deep buzz of this fly which in our experience greatly enhances its resemblance to the bumblebees. Its size is somewhat larger than its model, ranging from 23–29 mm. in length in a series of 86 specimens from Florida (Bromley, 1925).

During the course of research at the Archbold Biological Station during July, 1959, we became interested in the mimetic relationship of this asilid which we found abundant in a scrubland cattle pasture about four miles south of Highlands Hammock Park in Highlands County, Florida (Lat. 27° 25′ N.; Long. 81° 30′ 55″ E; U.S.G.S., Crewsville Quadrangle). In this tract of several acres one plant, *Petalostemon feayi* Chapman, was at the height of its flowering season and proved to be exceedingly attractive to many species of insects including *B. americanorum* workers. These latter apparently were in turn the chief attractants of *M. bomboides* which fed

exclusively upon them in this area. In addition to *M. bomboides* which is black and white, a black and yellow form much resembling it was present, which we have so far been unable to determine, as well as *M. nigra* Williston, known as the Black Bee Killer. The order of abundance of these three was roughly 40 white to 10 yellow to one black. We did not capture any individuals of two other species, *M. orcina* Wiedemann and *M. rex* Bromley, which also occur in Florida. In the middle of August, the whole complex of models and mimics began to decrease. This appeared to be due to the ageing of the Petalostemon flowers with a corresponding dispersal of the insects over a larger area. Our observations were conducted in this pasture about twice a week from 15 July–27 August, 1959.

As noted by Bromley (1930), *M. bomboides* has the habit of sitting on the stalks of tall weeds or on the tips of shrubs from which in our experience they launch their attacks. In the pasture it was usual to find about three asilids within one's view from any single vantage point. Each would be sitting on a stalk in a vertical position usually between one and three feet from the ground. Preference was shown by the flies for the shady side of the slender plant stems around which their large compound eyes could readily see. In filming these flies, we noticed that as the day progressed they shifted their positions so as always to be on the side opposite the sun. Linsley (*loc. cit.*) has also noted this preference for shade in *M. bromleyi* Curran in the southwestern United States. We estimated that at most the flies comprised 20 per cent of the combined populations of bumblebees and asilids. For every three of the asilids, there were about twelve of the bumblebees spaced out so that one fly would be near one clump of the lavendar flowers of Petalostemon, and another near a second some feet away.

In attacking, a fly very rapidly approaches a bumblebee from above and behind, usually just after the bee has left a flower, and grasps the dorsum with its long legs. It then immediately draws the bee towards its body, inserts its mouthparts and injects a substance which apparently kills or paralyzes the bee almost instantaneously. The fly then returns to a stalk, often the same one from which it began the attack, and proceeds, again in a vertical position, to digest externally the prey, a process which takes about five to ten minutes to complete. Afterwards the empty exoskeletons are sometimes left adhering to the plant stems. Many of the attacks were not successful, even if the fly touched the bumblebee with its feet, and when this was so, the fly resumed a perching position to await a later opportunity. It appeared to us that the contact had to be precise or else the fly would terminate its attack.

The rapidity of the effect of the injected poison almost certainly indicates that it is a neurotoxic substance, but its nature is unknown (Imms, *loc. cit.*). Whitfield (1925) has shown that it is secreted by modified thoracic salivary glands, whereas the saliva for external digestion is produced by special glands in the labium.

Copulating pairs of flies were often observed in which one partner was feeding on a bumblebee. We hope to investigate this behavior further to discover if the presentation of food by the male is a necessary part of the courtship ritual to prevent his being cannibalized by the female as is so in some of the similarly predaceous flies of the family Empidae (Imms, *loc. cit.*). Cannibalism of the male by the female has been noted in other species of Asilidae (Poulton, 1906) and the elaborate courtship of *Heteropogon lautus* Loew (Bromley, 1933) strongly recalls that of many spiders in which the male is often attacked by the female (Gertsch, 1949; Bristowe, 1958).

## LABORATORY INVESTIGATION OF MIMICRY

### *Materials and methods*

In order to test the hypothesis of Batesian mimicry of the bumblebee, *B. americanorum*, by the asilid fly, *M. bomboides*, experiments were carried out in which caged toads (*Bufo terrestris* Bonnaterre) were used as predators. Known as the Southern Toad, this anuran is common in the southeastern United States and was abundant in the vicinity of the Archbold Biological Station, being particularly easy to catch in the evenings after a rain storm.

On 3 July, 1959, 14 toads were collected along a wet road and brought to the laboratory where they were confined singly in cubic cages 12 inches on a side. These were arranged linearly on a bench three feet from the floor in a quiet corner of a large laboratory. The bottom and back of each was made of plywood, the two sides and front of black plastic screening, and the top was a piece of glass which could slide out to allow access to the interior. Cardboard partitions separated the cages so that the toads could not see one another, and only the top and front afforded a view to the exterior. During all experiments the area was illuminated by means of two 150-watt bulbs on the wall four feet opposite the cages, at a height of seven feet. The laboratory also received daylight through a north-facing skylight. Each cage was equipped with a ¾-inch deep petri dish containing water.

Seven of the 14 toads ate consistently when given preliminary feeding tests with various species of dragonflies. An additional one was captured on 6 July which fed regularly on dragonflies in one day. Of these eight toads, four were males and four females and the sexes were divided so that half of each was experimental animals and the remaining half, control animals. The eight cages were arranged as follows: (1) Experimental (E-1), male; (2) Experimental (E-2), male; (3) Experimental (E-3), female; (4) Experimental (E-4), female; (5) Control (C-1), male; (6) Control (C-2), male; (7) Control (C-3), female; (8) Control (C-4), female.

In preparation for each day's series, conducted in the evening hours when the toads were naturally active, bumblebees (*B. americanorum*), asilid flies (*M. bomboides*), and dragonflies were collected in the vicinity of the Archbold Station or in the pasture mentioned above. With rare exception, only

living insects were used in the experiments, a day's collection being stored alive in glassine envelopes in a cold-room until the evening.

All experimental insects were presented by means of dangling them on a black thread in front of the toads. In this way, though restricted, they could fly and/or buzz in a relatively normal manner, whereas if allowed to be free in a cage, they might alight out of reach of the toad. The dragonflies were tied by the posterior tip of the abdomen, and when a toad attacked, this broke readily. The bumblebees and flies were tied by the distal end of the third right leg and in most instances, the thread would slip off or the leg would break when a toad seized the insect. If this did not happen, the experimenter placed two fingers against the toad's mouth and pulled the thread free. This manipulation did not appear to alarm the toads, for they continued to feed freely after such treatment.

*Experimental design*

The design of the experiments was based on earlier mimicry studies (J. Brower, 1958, 1960) with some new modifications. Because dragonflies were found to be highly acceptable as food for the toads, one species *Pachydiplax longipennis* (Burn), commonly called the Blue Pirate, was used as the non-mimetic edible insect to ascertain that the toads were always ready to eat and that a rejection of a model or mimic would not be due to lack of hunger. Both control and experimental toads received their insects one at a time and the two groups were treated identically except that the experimentals received edibles, models, and mimics, whereas the controls were given only edibles and mimics. To be sure that the toads could not learn what to expect from the order of presentation, edibles and models (or mimics) were given sequentially in randomized pairs, each pair termed a trial (J. Brower, 1958). One randomization was used for all eight toads and can be seen in figure 1. Unlike the previous work, the experimental insects served as the sole food given to the toads and it was therefore necessary to adjust the number of trials given to the two groups so that they would be eating approximately equal amounts of food. The control toads generally ate both mimics and edibles and were given one trial each day for 17 days, that is to say, they ate two insects per day. The experimental toads, on the other hand, were given two trials daily for 14 days and generally ate only the edibles, likewise two insects per day. The only exception to this was the fourth day when only one trial was given.

The four experimental toads were given randomized pairs of models and edibles for ten trials. From then on, mimics were substituted for 50 per cent of the models. This order of presentation of mimics and models was determined by a random number table with the five lowest figures in a series of ten representing the mimic trials. A total of three series of ten was carried out, but in the final one, the trials were terminated after the last mimics had been given to the toads, thus making a total of 27 trials each. All experimental toads received the same sequence which can be seen in figure 1. The four controls received a total of 17 trials each. All toads were

allowed 1½ minutes to accept or reject an insect and if it was not eaten by that time, it was withdrawn from the cage. Three categories of reaction to the insects were recorded: (1) *NT* designating that a toad did not touch an insect; (2) *A* meaning that a toad attacked but did not eat it; (3) *E* meaning that a toad ate the insect. These experiments were conducted from 12–27 July, 1959.

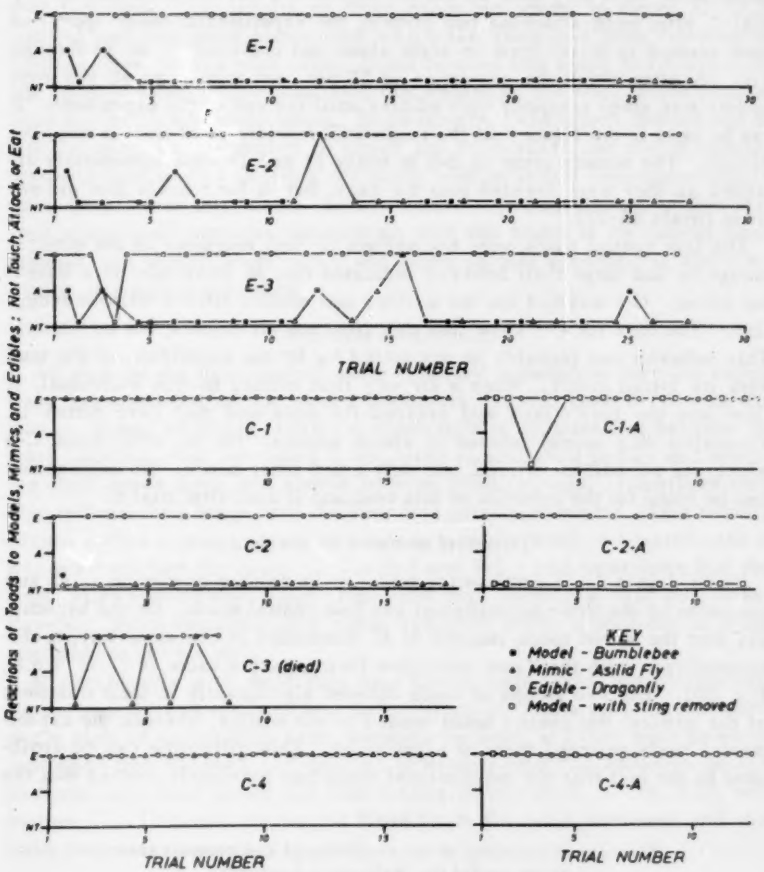


FIGURE 1. Reactions of experimental toads E-1, E-2, and E-3 to edibles, models, and mimics, and of control toads C-1, C-2, C-3, and C-4 to edibles and mimics. C-1 A, C-2 A, and C-4 A are the subsequent reactions of control toads C-1, C-2, and C-4 to edibles and models from which the stings had been removed. Each trial consists of two consecutive presentations, an edible and model, or edible and mimic. The asterisk above the first mimic in C-2 indicates that this fly apparently bit the toad's face (see text, p. 350).

### *Results*

The data for experimental toads E-1, E-2, and E-3 and control toads C-1, C-2, C-3, and C-4 are shown in figure 1. E-4 sporadically ate only three edibles and died on the ninth day and is therefore excluded from the results. Except for one instance, all edibles (139 out of 140) were eaten by the toads. The three experimentals attacked the model in the first trial. From this it seems likely that either the toads had no previous experience with bumblebees, or if they had, the earlier experience was not remembered. By trial 7, after each attacking two models, the experimental toads appear to have learned to reject them on sight alone and continued to do so through trial 10, after which the proportion of 50 per cent mimics to 50 per cent models was given randomly with edibles until the end of the experiment. It can be seen in the figure that the toads still rejected most models and also mimics. The mimics given to E-3 in trials 14 and 15 were immediately attacked as they were lowered into the cage, but in later trials this did not occur (trials 16–27).

The four control toads were not uniform in their reactions to the mimics, though by and large their behavior indicated that *M. bomboides* is a Batesian mimic. C-1 and C-4 ate the edibles and mimics offered without exception. The data for C-2 show that this toad ate all edibles, but no mimics. This behavior can probably be accounted for by the experience of the toad with its initial mimic. When a fly was first offered to this individual, it flew into the toad's face and grasped its nose and may have bitten it. Thereafter this animal refused to attack mimics. On the other hand, C-3 which ate all edibles offered, ate only every other mimic. No explanation can be found for the behavior of this toad and it died after trial 8.

### *Statistical analysis of the data*

Table 1 is a $2 \times 2$ contingency table of the number of mimics eaten and not eaten by the three experimental and four control toads. On the hypothesis that the seven toads reacted to *M. bomboides* in the same way, a chi-squared test, with the Yates correction factor, gave a value of 27.71, 1 d.f., $P < .001$. The two groups of toads differed significantly in their treatment of the mimics: the control toads tended to eat mimics, whereas the experimental toads rejected them on sight alone. This difference can be attributed to the fact that the experimental toads had previously learned that the

TABLE 1

Summary of reactions of the experimental and control toads to the asilid fly, *Mallophora bomboides*

| | Experimental toads | Control toads | Total |
|---|---|---|---|
| Mimics not eaten | 29 | 21 | 50 |
| Mimics eaten | 1 | 38 | 39 |
| Total | 30 | 59 | 89 |

bumblebee model was noxious, and did not distinguish between the bee and its mimic, an asilid fly.

*Experiments with bumblebees from which the stings were removed*

In order to determine whether the sting is the source of unpalatability in the bumblebee, *B. americanorum*, a second series of trials was conducted from 29 July–9 August with control toads C-1, C-2, and C-4 from the first series, now called, respectively, C-1A, C-2A, and C-4A. It will be recalled that C-3 died. The results of these trials are shown in figure 1. Only edibles and live bumblebees from which the sting had been removed by means of fine forceps were used. It can be seen that toads C-1A and C-4A ate 23 of 24 models. This indicates that except for the sting, these bumblebees are palatable to the toads. Interestingly, C-2A which had refused to eat the mimics also refused to attack the bumblebees, so that it appears that this toad was associating the model with an initial unpleasant experience (bite?) with the mimic. An alternative hypothesis could be that this toad remembered previous experiences with the model in its natural environment and rejected both mimic and model on that account. In either instance, mimicry is supported.

*Behavior of the toads*

All three of the experimental toads exhibited a particular reaction during many presentations of the model bumblebee, as did toad C-2A with the mimic. Noted by Noble (1931) as characteristic of defensive behavior in many Salientia, this consisted of the toads raising their bodies and lowering their heads down and almost between their forelegs. Sometimes they also inflated their lungs until the body was greatly distended. This usually occurred when a model came very close to a toad's head, and lasted until it was removed from the cage. Toads E-2 and E-3 would sometimes butt the model away with the dorsal part of the head, still keeping it held well down. However, these reactions were never exhibited before a toad had at least attempted to eat a model.

In experiments with *Bufo bufo bufo* Linné, Cott (1936) also mentioned similar reactions of toads to hive bees. He noted that a toad "...flinches at the sight of approaching bees, dropping the head, and then turns so as to face away from the bees..." (p. 119), or another toad "...crouches on the platform, with head bowed and chin resting on the wood..." (p. 121), and another "...flattened out on the board, with the head depressed and the limbs projected outwards." (p. 122).

## DISCUSSION

*Aggressive mimicry*

Several points have been raised by our field observations and by a review of the literature which lead us to consider the possibility of aggressive mimicry operating in the instance of the predaceous mimic asilid, *M. bom-*

*boides*, and its model bumblebee, *B. americanorum*. It has been seen that the adult flies prey to a considerable extent on their models, and although their mode of attack is relatively swift, the possibility exists that the mimetic resemblance of the fly enhances the likelihood of its being able to capture a bumblebee. A second level at which aggressive mimicry could operate is in the oviposition of asilid females in bees' nests. The food of *M. bomboides* larvae is not known, but Clausen (*loc. cit.*) noted that the eggs of the closely related *M. orcina* are said to be laid in small clusters in shallow holes in the soil and covered by the female. In general, Asilidae are predaceous as larvae, feeding on soft-bodied insects. Insofar as bumblebees often nest on or near the surface of the ground, under grassy tussocks, or in the abandoned nests of mice (Free and Butler, *loc. cit.*) it is possible that they could be exposed to the predation of asilid larvae. Aggressive mimicry might be operative when the mimetic female flies approach or enter the bumblebees' nest to lay eggs, the larvae of which would eat the bee larvae. Here again, mimetic resemblance of the fly to the bee might decrease the chance of a fly being attacked by a bumblebee. It is hoped that further observation and field experimentation will help to elucidate this problem and show whether a model can act as a selective agent in the origin and maintenance of the mimetic characters of its own mimic.

### *Batesian mimicry*

The experiments described in this paper demonstrate that the asilid fly, *M. bomboides*, is a Batesian mimic of the bumblebee, *B. americanorum*, which is noxious to the toad, *B. terrestris*, because of its sting. Although this is the first quantitative study of bee mimicry, Carpenter (1921) in Africa noted that one monkey (*Cercopithecus* sp.) ate a wasp-like asilid (*Hoplistomerus serripes* Fabricius) with great caution, while another rejected it altogether. However, this second monkey readily ate a non-mimetic asilid and these findings led Carpenter to suggest that the asilid was a true Batesian mimic. Similarly, Lloyd Morgan (1896) discovered that a Moorhen chick (*Gallinula chloropus* Linné) which had eaten and presumably been stung by a bumblebee thereafter rejected others from which the sting had been removed, as well as mimetic droneflies. Another inexperienced chick freely accepted both. In addition to providing evidence for Batesian mimicry, this experiment suggested that the noxious quality of bees is their sting, a hypothesis which was strongly supported by Cott (1936) in an extensive series of feeding experiments with toads and hive bees.

In order that Batesian mimicry should evolve and be maintained, several prerequisites are usually deemed necessary. First of all, the prospective model and mimic must occur together. Poulton (1904) has pointed out that insofar as mimetic asilids attack their aculeate Hymenoptera models, they seek places where these abound, thereby assuring sympatry. A second requirement is that models outnumber their mimics. Thirdly, the models must possess a noxious quality and advertise this by means of conspicuous

coloration. Each of these conditions is fulfilled by the *M. bomboides–B. americanorum* complex in the area which we investigated in south central Florida.

The use of *Bufo terrestris* as a caged predator in these experiments might be subject to criticism on the basis that it is not a major natural predator of bees and asilids. Toads become active at twilight and forage well into the night (Conant, 1958) and as far as is known lack color vision (Walls, 1942). Both of these factors make it unlikely that they acted as the main selective agents in the evolution of the mimicry of *B. americanorum* by *M. bomboides*. This is not to say that toads never attack these insects in their natural environment, particularly insofar as burrowing in the ground is an activity common to toads and bumblebees, and perhaps also to this asilid. What was important to know is whether toads can learn to reject bumblebees on sight alone and confuse them with mimetic flies. This has now been demonstrated.

## SUMMARY

1. Laboratory experiments described in this paper support the hypothesis of Batesian mimicry of bumblebees (*Bombus americanorum*) by asilid flies (*Mallophora bomboides*). Seven Southern toads (*Bufo terrestris*) were used as caged predators.

2. Experimental toads which initially attacked bumblebees learned to reject them on sight alone. They then also rejected mimics to a significantly greater extent than control toads which did not have prior experience with the bumblebees.

3. Two of the three control toads also freely ate bumblebees from which the sting had been removed. This indicates that the noxious quality is the sting.

4. Field observations of the predatory behavior of the adult flies in south central Florida show that the mimetic flies prey extensively upon their bumblebee models.

5. The idea of aggressive mimicry, proposed but then discarded by Poulton, is reconsidered in the light of new evidence and it is concluded that the selective basis for the resemblance of bees by flies, in addition to being Batesian mimicry, may in part also be the visual selection resulting from the defensive behavior of the models towards the mimics which attack them.

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# PREDICTION OF POPULATION GROWTH FORM IN *DAPHNIA PULEX* CULTURES*

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A variety of theoretical models forecasting population numbers have recently been suggested (Leslie, 1945, 1948; Slobodkin, 1953; Ricker, 1954; Nicholson, 1954; Wangersky and Cunningham, 1956, 1957). All include cases in which fluctuations in numbers occur in single species populations existing in an environment that may be constant, except for effects produced by the members of the population. Numerous observations on a variety of animals, which do not yield stability where this would be expected from a logistic model, have stimulated interest in schemes that provide for numerical fluctuations. Typical examples of such observed populations are flour-inhabiting insects (Park, Gregg and Lutherman, 1941) and Daphnia (Pratt, 1943; Frank, 1952; Slobodkin, 1954). For these, the logistic curve is clearly inappropriate (Smith, 1952).

Hutchinson (1948), Slobodkin (1954) and Wangersky and Cunningham (1956, 1957) have emphasized particularly the role of various lags in producing instability. Lotka (1925), in his derivation of the logistic, treated this curve as a special case of growth of a homogeneous population. He did not develop other cases, where the members of the population differ in mass, in any detail. Since animals grow after birth, their effect on and response to the environment will vary, often significantly, with age and size. Many of the newer models recognize this property of organisms. These models are demographic in that they take into account structural features of a population.

An ideal demographic model requires information answering these questions: (1) What are the significant classes in the population? (2) What are initial numbers in each class? (3) What are the birth, death, immigration and emigration rates for every population class in each environment to which it is subjected? (4) If the model is to predict energetics, what is the biomass and rate of production of members in each class? (5) What happens when the environment changes? This environmental effect can not necessarily be predicted by interpolation between constant, but more extreme conditions. If a time lag exists because of a change, either in population composition or in external environment, how long is the lag, and what parameters does it affect? At what rate and to what extent does each class adjust?

In natural populations the effects of numerous extrinsic variables, mainly associated with weather and other species of organisms, must be known in detail. We must know how to predict changes in these extrinsic factors. In

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some cases they will themselves be functions of the population whose numbers are to be predicted. There is no hope that we shall ever be able to make predictions at the level of this ideal demographic model for any but a few special situations. However, it is often possible to make reasonable estimates of population size, and short time projections based on these estimates and on natural history features are often of practical value. Improvements in these predictions will result from increased knowledge of what information will be most essential for success in such a venture, and which of the ideally desirable data are of less import, so that they may be ignored in a first approximation. A laboratory model can not provide the solutions to the general problem of prediction. It may, however, have considerable value in clarifying the role of factors that might otherwise be overlooked.

Population growth form has been characterized in the laboratory for three species of Daphnia, cultured under somewhat different conditions (Pratt, 1943; Frank, 1952; Slobodkin, 1954). The performance of these populations is at least grossly similar. Many of the vital statistics appropriate for testing models have been determined (Frank, Boll and Kelly, 1957). Thus one can delineate alternative predictive schemes, and contrast these with observed population growth. Even here, compromises between the desirable information and the practical difficulties involved in gathering it must be made. Every compromise damages the models somewhat. At best the empirical information permits one to make a slightly distorted prediction. The various departures are cumulative, so that a particular representation of a mathematical model does less and less justice to it the longer the period of prediction.

Study of the correspondence between models and observed population growth helps to define limits to the information that is critical. Inappropriate models are thus useful in providing clues to the effect of ignoring known variables. By making simplifications, it may be possible to indicate where, in future work, observations need to be made with care, and where cruder estimates will suffice. The contrasts between alternative theories, in this investigation, lead to rather unexpected information about the relative importance of the parameters that enter into our estimates. To what extent such information can be generalized is not known. Ultimately we hope to extend theoretical aspects of this study by determining minimal criteria for adequate representation of various features of growth form.

I should like to acknowledge the help of E. Novitski, who, in a number of discussions, helped in my formulation of the various models. I am also indebted to L. B. Slobodkin and F. E. Smith, who made useful comments on this manuscript.

## DATA FOR PREDICTION

The fundamental data specify size, growth, birth and death rates for all ages of Daphnia over a variety of constant densities that encompass what is expected in a growing population. This information, as well as the hus-

bandry methods used in the present investigation, have been published (Frank *et al.*, 1957). To supplement the constant density data, a number of cohorts were observed throughout life as in previous work, except that density was varied systematically once during the life span. Shifts over a wide density range from four to 24 animals per cu. cm. and vice versa represent the most extreme conditions. In more meaningful shifts over smaller ranges, animals at initial densities of 16 and 32 per cu. cm. were changed to half these densities, and animals kept at eight and 16 per cu. cm. were shifted to twice these densities. Duplicate sets of six cohorts were observed in all cases, and changes were made at one of two ages, 12 or 24 days. It would have been desirable to discover the effect of changes in degree of crowding on younger animals. However, it has become evident that the density conditions under which very young animals live are not comparable to those for older and larger ones, so that such an experiment would require crowding young animals with older ones. This is not feasible because fertility of the adults is too high.

Except for exaggerating the effects of change, data for the wide-range density shifts do not differ from the rest. Because these shifts are much more abrupt than changes that occur in the population, their significance is small, and the data will not be presented in detail. The results for the smaller range shifts do not vary appreciably with age of the cohort. When the animals are older, changes yield less reliable data because of the greater number of deaths that have occurred.

Figure 1 summarizes information from animals that have undergone a density change when 12 days old. Values for age specific survival ($l_x$), natality ($m_x$) and individual size ($v_x$) at each of three constant densities are indicated by dotted lines for purposes of comparison. Curves for cohorts in which density is increased are shown by interrupted lines; decreased density cohorts by solid lines. Arrows mark the time at which density was changed. The $v_x$ curves have been fitted by eye.

Increased density provokes an immediate response of decreased survival. The higher mortality rate declines to normal after four days; subsequently, death rates do not differ materially from those of equally old animals that have lived at the new density all their lives. When degree of crowding is reduced, death rates at once become indistinguishable from those at the lower constant density. Since variances of mortality rates are relatively large, particularly at ages greater than 20 days, one is unlikely to find significant differences. Changes in density do not affect natality so quickly. After a shift, no change in reproductive performance is detectable for two to four days. Thereafter the rate adjusts and becomes that characteristic of the new density once a minimum of six and a maximum of eight days have passed. Growth shows yet a different response. Not only is compensation to density change immediate, but overcompensation occurs. When density is lowered animals grow faster, and when it is increased they grow more slowly than would be expected of Daphnia of that age at the new density. These results are similar, in general, to those of Anderson, Lumer and

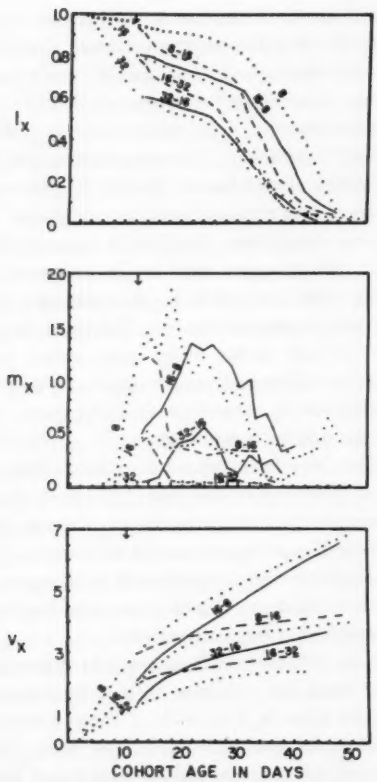


FIGURE 1. Effect of changing density on survival ($l_x$), natality per female day ($m_x$) and individual size ($v_x$). Density was changed at a cohort age of 12 days.
——— Density has been decreased by 50 per cent.
– – – – – Density has been doubled.
····· Constant density controls.

Zupancic (1937) and of Frank (1952). Accordingly, animals of a given age and density need not have the same birth, death and growth rates unless they have had the same prior density experience. Whether animals of the same age and size will have constant vital statistics, as postulated in the model of Slobodkin (1953), is not certain from our data, but it seems improbable.

Each of the three variables thus reacts somewhat differently to changes in crowding. With some exceptions, which are immediate aftereffects of the density change, mortality rates compensate well. Natality adjusts rather precisely, but only after an initial period of lag. Growth rates overcompensate, particularly shortly after density changes. Even the variations called "small range" here are more abrupt than the changes in degree of crowding occurring in experimental populations. Large range shifts, that is, abrupt changes from four to 24 animals per cu. cm., produce some results that are

absurd. For example, under these conditions mortality rate during the first two days following the density change increases to 0.75. In populations where mortality rates have been observed under conditions similar to ours, there is no indication that death rates ever reach such levels (Slobodkin, 1954). This is therefore certainly a spurious effect so far as growing populations are concerned. Although all our density shifts probably exaggerate the effects of such changes, they are probably not negligible in the populations to be predicted. However, a model accounting for the responses with precision would be exceedingly complex. The mortality effects are relatively minor and have been ignored in all our models. The natality lag could be incorporated into certain predictions in simplified fashion. The growth overcompensation proved troublesome, and the compromise made is not altogether satisfying, although it has the merit of being tractable.

## PREDICTIVE PROCEDURES

Predictive estimates have been made using six different models. Only one (Model C′) of these was expected *a priori* to be suited to the populations studied. All others ignore known variables. However, the rest of the models were applied in an effort to discover precisely what the effects of such simplifications are. The models may be subdivided into one group (A–D), in which particular values of the age and density specific vital statistics are determined by numerical density, that is, the numbers in a population in a constant volume (25 cu. cm.) of medium; and another (B′, C′) in which the total volume of the animals specifies the magnitude of the density effect. The first category disregards differences other than age between the members of the population at any one time. In the second, density becomes indirectly dependent on the age structure of the population.

A. The logistic curve. Constants needed for this model were derived from values for the intrinsic rate of natural increase (r) at various densities, as previously described. Values for the predicted population numbers, $N_t$, were obtained from the density units given in the earlier paper by multiplying by 25, the volume of the experimental habitats in cu. cm.

In this connection, it is necessary to point out that the values for "instantaneous birth rate" (*b*) in the earlier report are incorrect, as noted by Smith (1958). The data for *b* are, in fact, values of $\beta$ (see Andrewartha and Birch, 1954, pages 44 and 47 for notation and the relation between these statistics). As a result of this error the death rates *d* are also incorrect (figure 3, table 4, Frank *et al.*, 1957). The logistic estimate is, however, unaffected, since *r* is correctly presented.

For the remaining models certain terms, some of which have been discussed more fully in the earlier report, are essential:

1. Variables in the predicted population
   - N Total numbers
   - n Numbers in one age class
   - V Total volume of animals
   - w volume of one individual Daphnia

2. Subscripts, *etc.*
   - x Age interval from x to x + 2 days
   - t Population age in days
   - $\alpha$ Age at beginning of reproductive period
   - $\omega$ Age at end of life span (arbitrarily set at 52 days)
3. Vital statistics at constant density (from Frank *et al.*, 1957)
   - v Individual volume in cu. mm.
   - s Growth rate of an individual: $\frac{v_x}{v_{x-2}}$
   - p Survival rate: probability that an animal will survive during a given two day interval
   - m Birth rate: young produced per female in two days.

The data for predictive estimates consist of:

1. Initial values of the numbers and volumes of the members of the population for every age class.
2. Information about the time lag in vital statistics after changes in density.
3. Tables of v, s, p, and m at seven constant densities (1–32 per cu. cm.) and at all ages (Frank *et al.*, 1957).

Values for densities not given in the tables were estimated by linear interpolation. Extrapolation beyond the highest numerical density (32 per cu. cm.) was required in certain predictions at the population peaks. The assumptions were made that at a numerical density of 64 per cu. cm. birth rates were half, and death rates twice those at the highest observed density; growth rate was assumed to undergo no change. These assumptions are undoubtedly incorrect; however, large departures from them would be required to affect the predictions significantly.

Model B is similar to that of Leslie (1945). The population is defined by the relations:

$$n_{x+2,\,t+2} = n_{x,\,t}p_x(N_t)$$

$$n_{0,\,t+2} = \sum_{x=\alpha}^{\omega} n_{x,\,t}m_x(N_t)$$

$$N_{t+2} = \sum_{x=\alpha}^{\omega} n_{x+2,\,t+2} + n_{0,\,t+2}$$

Parentheses are used to characterize functions, and do not denote multiplication.

As in the remaining predictions, successive estimates of population numbers are got by tedious evaluation for each time interval in turn. The values at a population age of t + 2 days are calculated from known values at t days by considering the death rates that apply to each age class at that numerical density $N_t$, and by summing the births contributed by each age class. Prediction B differs from the logistic because (1) it uses discrete population growth intervals, and (2) it considers the effect of different initial age distributions. It ignores lags at the level of the individual. Model B′ differs from B only in its criterion of density, which is volume. The equations for B can be readily modified by substituting $(V_t)$ for $(N_t)$ on the right side of the equations for Model B. However, the vital statistics data now apply

to volume densities, and V must be calculated for the population in addition to N. Volume of an animal was assumed to follow the growth rate appropriate to that age and volume density, with one exception: if an individual had already attained the absolute size characteristic of a given age at a certain density, the assumption was made that the animal would remain the same size.

$w_{x+2,t+2} = w_{x,t}s_x(V_t)$, except if $w_{x,t}s_x(V_t) \geq v_{x+2}(V_t)$, in which case $w_{x+2,t+2} = w_{x,t}$.

$$w_{0,t+2} = 0.15$$

$$V_{t+2} = \sum_{x=0}^{\omega} w_{x+2,t+2}{}^{n}x+2,t+2 + w_{0,t+2}{}^{n}0,t+2$$

Thus the increased growth rate that occurs when crowding is reduced is not accounted for; the decrease in growth rate at increased density is rather crudely approximated. Model B′ can be considered more realistic than B, since it takes into account the different crowding effects that animals of different sizes can be expected to have. However, because it treats birth rates as if they adjusted immediately to the population's density, it also ignores known information.

Predictions C and C′ correspond to B and B′ respectively, but include the observed natality lag in their formulation. This lag was estimated to average five days. Therefore in C and C′ the effect of density on birth rate was assumed to be that of the population that existed five days prior to a given population age. A simple modification in the formulas is all that is required: wherever $(N_t)$ or $(V_t)$ occur in B or B′, $(N_{t-5})$ or $(V_{t-5})$ must be substituted. This device is equivalent to assuming that birth rates are those characteristic of a certain density that existed five days previously, and then shift abruptly to a subsequent density. Actually there is a certain rate of accommodation during the lag interval. This has been ignored.

Model D, developed by Ricker (1954), was applied by him with surprising success to Pratt's (1943) data. It assumes that there is no further adjustment to density change once an animal has reached maturity. This prediction and the logistic may therefore be interpreted as opposite limiting cases of populations regulated by numerical density. The logistic presupposes instantaneous reaction of the population to density change; Ricker's formulation assumes maximal lag. This model requires values of adult numbers in successive generations at any one initial density. These values may be calculated, in our example, from existing vital statistics data (table 1). The table presents calculated generation length at each density as well as values for the net reproduction rate $R_o$ (Andrewartha and Birch, 1954), and population numbers expected in successive generations. Ricker's model makes no allowance for the existing differences in generation length, although it could, of course, be modified to include them. For details regarding the features of this model, the original paper (Ricker, 1954) should be consulted. We used six day intervals for successive estimates of the

TABLE 1

Statistics for Ricker's model (D)

| Density No./cc. | Net reproduction rate per generation $R_o$ | Numbers in generation | | Generation length, days T |
|---|---|---|---|---|
| | | i | i + 1 | |
| 1 | 39.3 | 25 | 983 | 13.2 |
| 2 | 41.0 | 50 | 2050 | 13.6 |
| 4 | 42.8 | 100 | 4278 | 13.8 |
| 8 | 26.4 | 200 | 5286 | 17.1 |
| 16 | 5.9 | 400 | 2365 | 19.2 |
| 24 | 0.48 | 600 | 288 | 18.2 |
| 32 | 0.13 | 800 | 101 | 27.7 |

Column 2 is derived from age specific birth and death rates in Frank *et al.* (1957). Column 3 gives the numbers per cohort, and equals column 1 times 25. Column 4 is the product of columns 2 and 3. The last column may be estimated from known data, as in column 2.

population, as did Ricker for Pratt's data on *D. magna*, since the refinement of finer intervals did not seem merited.

## COMPARISON OF PREDICTION AND OBSERVATION

The populations against which various models are tested include 12 replicates started with 25 animals in 25 cu. cm. of medium. In these, the stable age distribution for this density was approximated as closely as was possible. Six other populations initially had 400 Daphnia (200, 3–5 days; 100, 9–11 days; 50, 15–17 days; and 50, 21–23 days old). The days on which different replicates were observed were staggered by groups of three replicates, so that extrinsic factors that might have escaped control could be evaluated. All populations were censused at two-day intervals for 120–130 days.

When the models are applied to the populations with an initial 25 Daphnia, the curves of figure 2 result At first glance, the large differences between the several predictions, although based on the same vital statistics, stand out. The most cursory examination reveals that certain models must be inadequate because of the discrepancies that exist between the predictions. It is profitable, for the moment, to compare the models further without having recourse to what is revealed by the observed populations.

The logistic model (A) and Prediction B are very similar, except that the asymptote is attained significantly faster in the logistic. With greater deviation of the initial population from the stable age distribution, somewhat larger differences between these models would result. Both theoretical populations level off when the numbers of animals reach 582, although there are, in Model B, minor fluctuations about this value. When Prediction B is contrasted with B′, the major possible role of size differences is emphasized. Although the two models differ only in their criterion of density, numbers as compared with volume, maximum population size in B′ is more than twice that of B, and major fluctuations are introduced by changes of

size with age. This type of lag has been termed "populational" by Slobodkin (1954), as contrasted with "individual" lags, such as that in birth rate.

The natality lag produces similar, but smaller fluctuations with shorter period (Model C). Prediction C′ may be thought of as possessing the joint properties of B′ and C. Both populational and individual lags are considered. The two lags enforce each other, so that maximum population numbers are almost twice those of B′, and four times the asymptotic value of the logistic curve. Ricker's model yields a predictive curve that most resembles C′. Here a long individual lag, but no populational lag has been assumed.

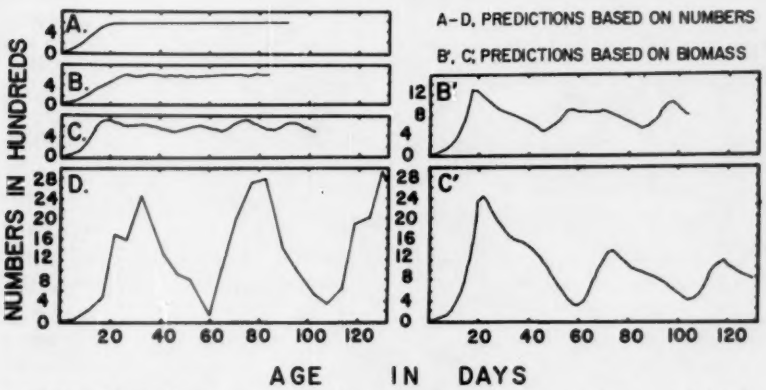


FIGURE 2. Predictive estimates of numerical population growth from an initial population of 25 Daphnia. Further explanation of the models in text.

Of the six models, only C′ makes use of all the known demographic information for Daphnia; it also contains some admitted distortions. It is gratifying that the growth form predicted by this model agrees rather well with that of observed populations. The six replicates of figure 3 provide a sample of the empirical observations. They have been successively displaced along the ordinate (by 400 animals) so that the individual curves will not obscure each other. Replicates 1, 2 and 3 were observed on the same days and received the same lots of food algae. The other populations were counted on alternate days. Some consistent differences are observable between these two subsets. However, in their general features the curves exhibit reasonable conformity. All empirical populations exhibit a peak of 2200–2550 animals between days 22 and 26. This is also true in the six replicates whose curves are not presented here. The timing and height of this peak agree well with Prediction C′. Observation and prediction are in general accord during the period of decline, roughly from day 22 to day 60. The second predicted peak is not realized fully in the observed populations. By this time the individual replicates are somewhat out of synchrony. However, even the third peak, predicted at 120 days, is roughly

approximated in all twelve replicates. This is surprising, since the model must be expected to predict more poorly over long than over short periods. Ricker's model (D) predicts the times of successive peaks as well as does C′, but the amplitudes of the later fluctuations are much too high. Since his model is known to depart in major respects from what is known about the nature of lags in our animals, the existing correspondences between D and the observed populations must be considered fortuitous.

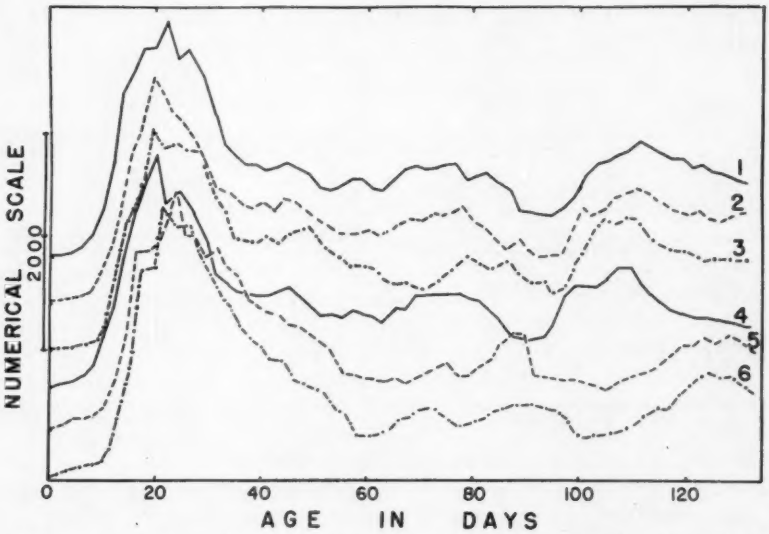


FIGURE 3. Numbers observed in six replicate populations with initial population of 25 Daphnia approximating the stable age distribution at that density. (The ordinate has been successively shifted by 400 for different replicates.)

Additional evidence that Model C′ yields a fairly good predictive estimate is provided by applying it to populations with an initial 400 animals. Comparisons between a single representative curve, Prediction C′, and that of Ricker (figure 4) indicate that C′ is fairly but not completely satisfactory. The initial peak is reached significantly faster in the predicted curve, but the agreement between prediction and observation is as good as may reasonably be expected, considering the liberties that were taken with the data. Ricker's model does not provide a good fit, although again the departures are primarily in amplitude.

Another test of Model C′ involves the dynamics of population change. The model actually predicts population size, total birth and death rates, and population volume. Unfortunately our observations provide no information on biomass, and yield only semiquantitative evaluations of birth and death rates in the populations. These have been estimated from changes in total population size, and the number of small animals noted at time of

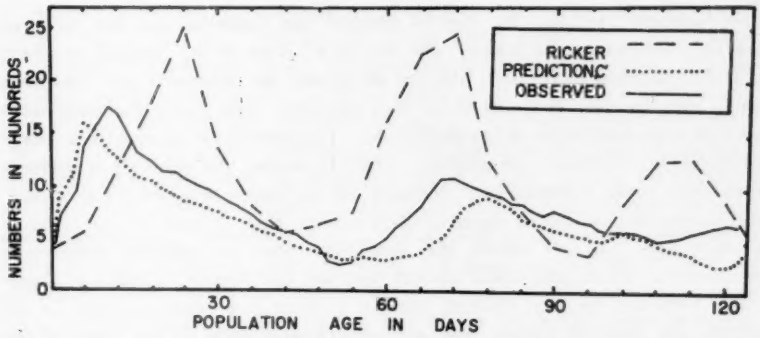


FIGURE 4. Comparison between the prediction of Model C′, D, and an observed population started with 400 Daphnia.

census. What happens in the model is in general agreement with our own observations, and those of Pratt (1943) and Slobodkin (1954). At the beginning, total birth rate is high and death rate is low. As the population increases in numbers, and as the individuals become older, birth rate declines as the result of the increased density, and because of the changes in age structure. At the population peak, births essentially cease. This prediction of the model holds for our populations. Death rate subsequently increases, but has once again declined at a population age of 30 to 40 days. This is purely the result of age structure: at this time the great majority of animals belong to intermediate age classes whose age-specific death rates are low even at high densities. After day 40, the predicted population once again decreases more rapidly, since now the animals are so old that their death rates are high. Up to this point, no renewed spurt of natality has occurred. This can be explained when one notes the predicted biomass. It does not reach its peak until about day 40, despite the considerable decline in numbers that has occurred by then. The decline is more than compensated by additional growth of the animals that remain. Only after day 40

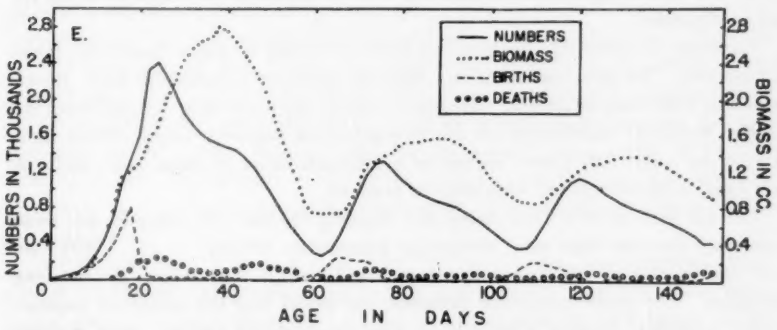


FIGURE 5. Population dynamics of Daphnia according to Prediction C′.

does biomass decline. By day 54 natality has risen so that the increase can be observed in the graph. By this time, most of the original members of the population have died, and the remainder are relatively old, and have small age-specific birth rates for that density. The cycle therefore does not attain the amplitude of the earlier one. Whenever new increases in population occur, numbers rise slightly before biomass. As population numbers pass their peak, biomass has, however, by no means reached its maximum, and a populational lag of about 18 days results from growth of the animals as they age. As net result, the population does not exhibit continuous generations, which one would expect from the life history features of Daphnia. Although the reproductive portion of their life span can include all except the juvenile stages that occupy approximately 15 per cent of the total life span, this reproductive capacity is not realized under our conditions of culture. Since natality varies so widely with changes in density, and individual growth continues when density is so high that reproduction is effectively nil, successive generations become almost discontinuous.

## DISCUSSION

This picture of population dynamics differs only in minor regards from earlier analyses of this and other species of Daphnia. Likewise the most satisfactory model is closely related to that of Slobodkin (1953), which was intended to be applicable to his populations of *Daphnia obtusa*. Appropriate data to test this model are not available. However, the relationships between the two models are sufficiently close that there is no reason to question that it would be suitable, given the proper limiting factors and sufficient empirical information. Both formulations are iterative, and can therefore be readily modified to accommodate additional information, such as the observed birth lag. Mathematically, they are rather inelegant. In Slobodkin's prediction, given a known energy input, age and size classes define values for birth, death and growth rates. In mine, time and volume density, acting on a population of specified age and size distribution, cause the vital statistics to take on certain values that determine the population. Were we to assume that energy input is limiting for our animals, the models could be made identical.

Attempts to identify the limiting factors in our Daphnia have not been completed. We are hoping to be able to correlate information from these animals with that of others. Richman (1957) and Slobodkin (1959) have obtained valuable information on the energetics of *Daphnia pulex*. Their data are for animals with a food régime of a different order of magnitude, and can not readily be integrated with ours at present.

Certain indications concerning the limiting factors operating in our system were derived from vital statistics information (Frank, *et al.*, 1957). At low densities a different density effect from that at higher levels of crowding obtains. The correspondence between our model and the observed populations provides another clue. It is not immediately evident why volume should provide a proper measure of whatever density means in a physiologi-

cal sense. Yet there is virtually no doubt that the volume density model provides more than an accidental fit to our populational data, since there are so many independent areas of correspondence between them. We can therefore assume that volume density provides a suitable substitute for some variable that has the observed effects on vital statistics here attributed to density. Slobodkin (1954) has generalized about the role of crowding in Daphnia. He states that, in all known cases, density effects are indirect: results of interactions between animals and their external environment, rather than directly between animals. It is instructive to note that Richman's data for respiratory rates of small and large animals show about the same range of variation—about 15 fold—as does volume among our relatively crowded animals. This is not true of his dry weight measurements, which vary much less. Requirements used or metabolites produced by Daphnia are probably simple functions of respiratory rate. Thus it is likely that the limiting factor under population conditions is an indirect density effect. By chance, volume is sufficiently closely correlated with whatever causes the results attributed to density that it functions well enough in our predictions.

Comparisons between the models permit some evaluations of the relative contribution of different variables on the fluctuating growth form. The two relations that play the most important roles are those between births and density, and between age and size. None of the predictions that ignore size differences are at all indicative. This variable interacts with the rest, since it determines density. The effect of density on birth rate is characteristic of all models, and is the main reason for the attainment in them of an upper limit. Since such a labile birth rate is unusual, its general significance is small. Other density dependent variables could produce similar results. The effect of density change, particularly on natality, is far from negligible. However, the interaction between the population lag produced by size changes and the individual lag in birth rate is particularly noteworthy, and was unexpected. These two lags cause fluctuations that resemble those exhibited by Ricker's model, and could be represented by a model in which one lag is ignored and the other is exaggerated. A more general treatment of lags may perhaps be possible, since it is apparent that lags from several causes may be combined by a suitable function. The relation between growth and density has only minor effects on the shape of the population curve. Since Daphnia are able to adjust, not only are population peaks numerically higher than they would be without stunting, but the valleys are not so deep. As numbers decrease, additional growth of stunted animals occurs, and results in increased reproductive ability. Thus stunting may have a significant adaptive effect when it no longer is observable in the population.

The fluctuations in both models and populations are quite regular. Control of environmental variables is in part responsible. However, this regularity must be attributed in part to the short pre-reproductive period in the life span of Daphnia, since Ricker (1954) has demonstrated that, as this

period increases in populations with overlapping generations, increasing irregularities develop.

The demonstration that a demographic model provides a successful approximation of an observed population in a relatively constant laboratory environment leads logically to the question of the general utility of predictions of this sort. Iterative models can be constructed that allow for virtually all contingencies one may expect to arise in various populations. The mathematics present little difficulty, and solutions for specified initial conditions are tedious only so long as they are not assigned to an electronic computer.

Despite this, such models are unlikely to solve the practical problems of forecasting specific natural populations because they require enormous amounts of information. The data available for making our predictions could have been restricted in several ways without doing gross damage to the model. The age intervals for which death and individual growth rates were estimated are much finer than necessary. However, this is not true to the same extent for birth rate, since the exact age when reproduction begins is rather critical. The density intervals for birth and growth rates are finer than was required. This does not hold for survival, which varies with density in more complex fashion. However, the total role of variations in death rate with density is relatively small, and even had density effects on mortality been ignored altogether, this would have been serious only among the young animals, where death rate soars at high density. Effects of density change need have been estimated only for one age group and one set of two densities.

It is evident that these simplifications could be applied only in retrospect, and only to this particular model. One generalization can, however, be made. The significance of the vital statistics information declines greatly with individual age, so that once the peak of the reproductive period is reached, virtually any approximation will be adequate. Conversely, what happens to the young stages is of extreme importance, and estimates at these ages must be precise. This conclusion is also implicit in Cole's (1954) treatment of natural history features of populations, and certainly has wide validity. It is unfortunate, therefore, although not surprising from an evolutionary point of view, that vital statistics information is most difficult to gather precisely for those age classes that are most important.

This unoptimistic analysis is not meant to imply that demographic models are useless, but rather that their function lies in a different direction. Our understanding of the behavior of populations has advanced considerably during recent years, largely as the result of increased attention to structural features. Bodenheimer's observation (1938) that the study of age distribution represented one of the most neglected areas in ecology is no longer so applicable today, although there is still a dearth of empirical information along these lines. Models simulating what is known qualitatively about various single species populations and about interactions of different types are likely to provide significant insights into the range of possible events

in these systems. They may indicate observable entities that characterize some particular process. Thus they function partly to clarify what might otherwise be vague and seemingly intuitive ideas. They gain additional value whenever they can predict populational features that can be verified empirically. These predictions are unlikely to be specific and quantitative, since we rarely have enough information to estimate precisely the constants that enter into particular formulations. One may reasonably hope that demographic models can furnish useful generalizations. Most likely, however, theory and empirical observation will continue to advance concurrently rather than sequentially.

## SUMMARY

Available data on age- and density-specific birth, death and individual growth rates, combined with new information on lags in these statistics following density change, can be applied to models of population growth of Daphnia in a constant environment. Six alternative models have been tested. They range in complexity from the logistic curve to an iterative model that takes into account the initial age distribution, and effects of age, density and density change on natality, mortality and size. This complex model is the only one that provides a fair representation of observed events in experimental Daphnia populations. Several independent areas of agreement between observations and model provide assurance that the observed fit is not merely fortuitous.

The most important relations determining population growth form in this system are those between (1) natality and age, density and density change and between (2) size and individual age. Comparisons between the models emphasize that, in most organisms, the populational effects of different age and size classes can not be considered negligible in assessing population dynamics. Knowledge of the vital statistics of the younger members of a population is of paramount importance for demographic prediction.

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# THE MECHANISM OF NATURAL SELECTION FOR THE SEX RATIO

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The sex ratio of many species is under genetic control (Darwin, 1871; Crew, 1937; Mayr, 1939). Darwin (1871) felt that if the sex ratio were under genetic control it should be fixed by the mechanisms of evolution, but he could see no mechanism by which natural selection could affect the sex ratio and preferred to leave the problem to the future. R. A. Fisher, in *The Genetical Theory of Natural Selection* (1929), stated "that the action of Natural Selection will tend to equalize the parental expenditure devoted to the production of the two sexes." The possible genetic mechanisms involved in the determination of sex ratios were discussed by R. F. Shaw (1958). The problem of selection for sex ratio was approached by Shaw and Mohler (1953), but the important relationship between expenditure and the sex ratio was not considered. This paper will present a population model which quantifies and extends the mechanism described by Fisher.

There is obviously a limit to the number of new individuals that can be produced by any single organism. This restriction may be imposed by one or more limiting factors, for example, the amount of food material available for the production of gametes, the amount of food available to feed the young, or the amount of space in the uterus. Now suppose that X equals the number of males that could be produced by any individual if only males were produced, and that Y equals the number of females that could be produced if only females were produced. These constants are represented by the points X and Y in figure 1. The size and structure of families consisting of both sexes are given by some function joining the points X and Y. If there is no

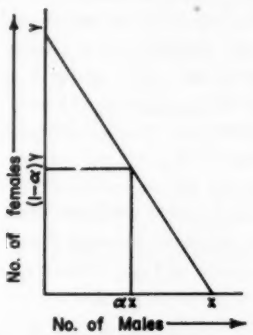



FIGURE 1. Illustrating the possible structures of families with regard to sex of the offspring.

interaction between the expenditures per male and per female, that is, if the expenditure per male or per female is the same irrespective of the number and sex of its siblings, then this function is simply the straight line shown in the figure. Consider a population of two mating pairs, A and B, whose total expenditures on the production of young are, for simplicity, unity. Now if the expenditures of A and B on the production of males are $\alpha$ and $\beta$, respectively, their expenditures on the production of females are, respectively, $(1-\alpha)$ and $(1-\beta)$. Pair A will produce $\alpha X$ males and $(1-\alpha)Y$ females, and pair B will produce $\beta X$ males and $(1-\beta)Y$ females. The proportion of males in the next generation derived from A is:

$$\frac{\text{No. of males from A}}{\text{Total males}} = \frac{\alpha X}{\alpha X + \beta X} = \frac{\alpha}{\alpha + \beta}.$$

Since each of the males derived from pair A inherited all of its genetic materials from the two individuals which constitute pair A, the genetic contribution of A to the total male population of the next generation is $\frac{\alpha}{\alpha+\beta}$. By the same reasoning, the genetic contribution of A to the total female population of the next generation is $\frac{(1-\alpha)}{(1-\alpha)+(1-\beta)}$. Since every individual in a sexually reproducing population has one parent of each sex, each of the sexes contributes equally to the ancestry of future generations. It follows that the contribution of A to future generations is:

$$C_A = \tfrac{1}{2}\frac{\alpha}{\alpha+\beta} + \tfrac{1}{2}\frac{(1-\alpha)}{(1-\alpha)+(1-\beta)}. \tag{1}$$

If the composition of this population is stable the contribution of either pair to future generations is one-half, and if the contribution of either pair is not one-half the genetic composition of the population is shifting. Equation (1) and the conclusions which will be drawn from it, also hold for a population of any size, which is divided into two groups of equal size, A and B, which mate randomly within themselves, but between which there is no mating, whose mean expenditures on males are, respectively, $\alpha$ and $\beta$, and whose progeny will mate randomly in a single homogeneous population. $C_A$ is then the contribution of the entire group A to future generations. Similar equations, which differ from equation (1) only in the constants, can be written for populations divided into two groups of any size.

Figure 2 gives the values of $C_A$ for all values of $\alpha$ when $\beta$ is 0.1, 0.5, and 0.9. When $\beta = 0.5$ A can do no better than $C_A = 0.5$. But if B's expenditure deviates from 0.5, A can contribute more than one-half to the succeeding generations. The optimum strategy for A is that value of $\alpha$, $\alpha_{max}$, which gives the maximum value of $C_A$. Equating $dC_A/d\alpha$ to zero and solving for $\alpha_{max}$ gives:

$$\alpha_{max} = \frac{3\beta - 2\beta^2 \pm 2\sqrt{\beta - \beta^2}}{2\beta - 1}.$$

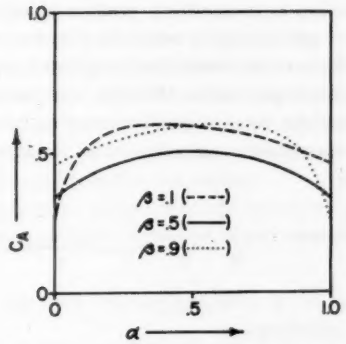



FIGURE 2. The contribution to future generations of pair A, $C_A$, for various $\beta$, the expenditure on males of pair B, as a function of $\alpha$, the expenditure on males of pair A.

Figure 3 gives the values of $\alpha_{max}$ for all values of $\beta$. As $\beta$ varies from 0.1 to 0.9 A's optimal strategy shifts from 0.4 to 0.6. By maintaining the strategy $\alpha = 0.5$ A can never be very far from gaining its maximum contribution, and can never gain less than one-half of succeeding generations. Since the equations are symmetrical for pairs A and B, the conclusions drawn for A also hold for B, and the population is stable if both pairs are investing half of their total expenditures in the production of males.

If a population is not expending equally on both sexes, any variations toward equal expenditure, or toward unequal expenditure in the opposite direction, will readily be passed on to future generations and the total expenditures on each of the sexes will tend toward equality. Since the expenditures per male and per female do not enter the equation, but the total expenditures on the production of males and of females do, this selection can be for total expenditure only and not for expenditure per individual or per pair. That is, the

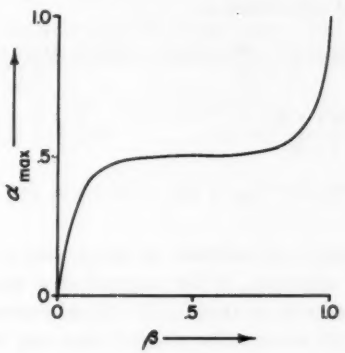
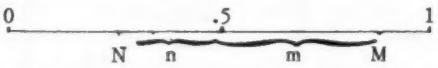



FIGURE 3. The values of $\alpha$ which give the maximum values of $C_A$, $\alpha_{max}$, as a function of $\beta$.

sex ratio of a population half of which produced only males and the other half of which produced only females would be just as stable as the sex ratio of a population in which every individual expended equally on both sexes. More generally, consider a population divided into two groups of M and N individuals. Suppose that the mean expenditure on males of an M individual is .5 + m and the mean expenditure on males of an N individual is .5 − n. This can be represented by:




If N/M = m/n = k, that is, N = kM and m = kn, then the contribution to future generations of an M individual is

$$C_{M_i} = \tfrac{1}{2}\left[\frac{\tfrac{1}{2}\text{ No. of males from } M_i}{\text{Total males}} + \frac{\tfrac{1}{2}\text{ No. of females from } M_i}{\text{Total females}}\right]$$

$$= \tfrac{1}{4}\left[\frac{(.5+m)X}{M(.5+m)X + N(.5-n)X} + \frac{(.5-m)Y}{M(.5-m)Y + N(.5+n)Y}\right]$$

$$= \tfrac{1}{4}\left[\frac{.5+kn}{M(.5+kn) + kM(.5-n)} + \frac{.5-kn}{M(.5-kn) + kM(.5+n)}\right]$$

$$= \tfrac{1}{4}\left[\frac{(.5+kn)+(.5-kn)}{.5M(1+k)}\right] = \tfrac{1}{4}\left[\frac{1}{.5M(1+k)}\right],$$

and similarly, the contribution to future generations of an N individual is

$$C_{N_i} = \tfrac{1}{4}\left[\frac{(.5-n)}{N(.5-n) + M(.5+m)} + \frac{(.5+n)}{N(.5+n) + M(.5-m)}\right]$$

$$= \tfrac{1}{4}\left[\frac{(.5-n)+(.5+n)}{.5M(1+k)}\right] = \tfrac{1}{4}\left[\frac{1}{.5M(1+k)}\right]$$

and $C_{M_i} = C_{N_i}$, therefore the population is stable. The mean expenditure on males for the total population is

$$\bar{E}_{\sigma} = \frac{N(.5-n) + M(.5+m)}{N+M} = \frac{.5kM - kMn + .5M + kMn}{M(1+k)}$$

$$= \frac{.5M(1+k)}{M(1+k)} = .5\,.$$

(It can also be shown that $C_{M_i} = C_{N_i}$ if $m = -n$, but this case can be considered trivial.)

Now, since the total expenditures on males and on females are fixed at equality by natural selection, if the expenditures per male and per female are constant, the sex ratio is also fixed. Equal expenditure, however, need not imply an equal sex ratio. The expenditures may be equal, but the numbers of the sexes unequal if the expenditure per male does not equal the expenditure per female, or if there is a differential mortality affecting the sexes

either pre- or post-natally. But a differential mortality can affect parental expenditure only up to the end of the period of parental care, so that any differential mortality after this point can have no effect on the sex ratio. Moreover, since the selection is only for the total expenditure, only the mean sex ratio is fixed and there is no effect on the variance, that is, a population can have any degree of heterogeneity so long as the totals expended on the production of each of the sexes are equal.

Research is in progress to investigate the extent to which this mechanism plays a role in the determination of the sex ratio in natural and in laboratory populations.

## SUMMARY

A population model is presented which quantifies and extends the mechanism described by R. A. Fisher for the natural selection of the sex ratio. It is shown how the sex ratio should adjust so that the total parental expenditure on the production of males is equal to the total expenditure on the production of females, and that this mechanism can affect the mean of a population's sex ratio, but not the variance.

## ACKNOWLEDGMENT

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# LETTERS TO THE EDITORS

Correspondents alone are responsible for statements and opinions expressed. Letters are dated when received in the editorial office.

## INCOMPATIBILITY SYSTEM IN *THEOBROMA CACAO**

The genetics of self-incompatibility in *Theobroma cacao* is considered unique among the identified systems in the flowering plants. Knight and Rogers (1955) reported that it has a sporophytic system controlled by one series of *S* alleles which show dominant or "equal" (independent) action in both male and female organs. However, pollen-tube studies showed that there is no inhibition of the pollen-tube growth in incompatible crosses and that the contents of the pollen-tube are liberated in the ovules, the ensuing failure to set fruit being due to a subsequent incompatibility. Further cytological studies by Cope (1958), revealed that in incompatible crosses, nonfusion of gametes may occur in 25, 50, or 100 per cent of the ovules. He proposed that the gametes that do not fuse in the embryo-sac are those carrying the same dominant allele. This gives the impression of a gametophytic control of incompatibility

To explain the unusual situation in *T. cacao*, Cope postulated the existence of two independent loci: one (*P, p*), which controls the production of an incompatibility precursor, shows simple dominance and recessivity and acts before meiosis; the other ($S_1, \ldots S_n$) imparts specificity to the precursor, shows allelomorphism and acts after meiosis. He suggested that the self-compatibility of the Central American and Trinidad populations of *T. cacao* is due to one or both loci being inactive ($pS$, $PS_f$ or $pS_f$ where f indicates the presence of an amorph). A cross between representatives of the above mentioned populations (believed to be $pS$ and $PS_f$) gave all self-incompatible trees.

The experimental results of Knight and Rogers and Cope, leave no doubt about their conclusion regarding the physiological nature of the incompatibility system in this species. However, the genetic interpretation of these processes is unnecessarily complicated. A precocious (premeiotic) action of the specific (*S*) pollen growth substance producing unit of the *S* gene complex, when interaction between the two *S* alleles could occur, has been postulated in the hybrid between the gametophytically controlled self-incompatible *Oenothera pallida* (♂) and self-compatible *O. trichocalyx* (♀) (Crowe, 1955; Pandey, 1960). In this hybrid the *S* allelic specificity is superimposed upon specific pollen growth substances post-meiotically.

The sequence of events, regarding *S* gene action, in *T. cacao* may be assumed to be as follows (figure 1): (1) premeiotic production of specific growth substances leading to *S* allele interaction, (2) post-meiotic production of specific incompatibility precursors, (3) conversion of specific precursors into specific incompatibility substances in pollen-tubes in the male

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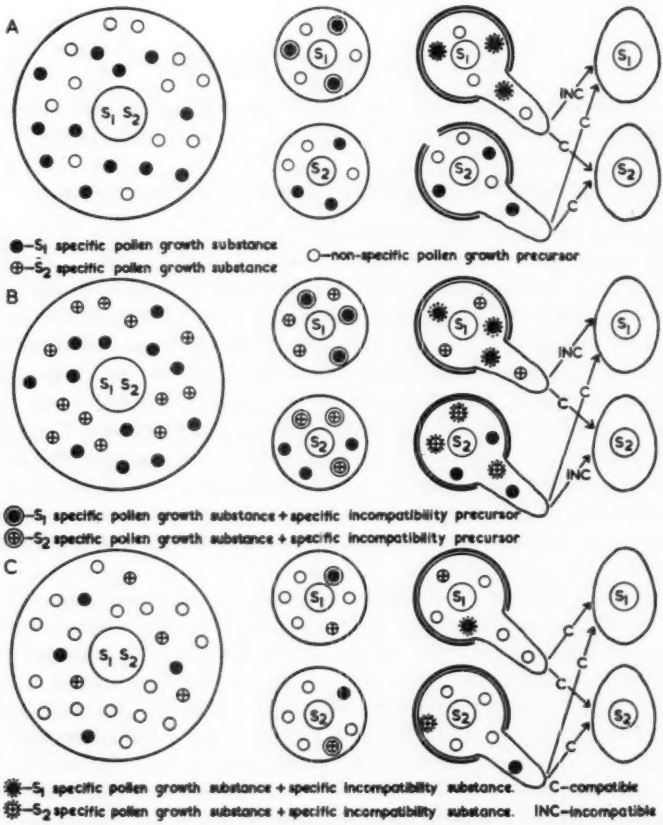


FIGURE 1. The suggested sequence of events regarding the physiology of *S* gene action in *T. cacao*. There may occur dominance interaction (A), independent action (B), or competitive interaction (C) between *S* alleles. For details, see text.

and in embryo-sacs in the female, (4) growth of all pollen-tubes down the style and into the embryo-sacs because of lack of incompatibility substances in the sporophytic, stylar and ovarian, tissues and (5) incompatible reaction between the pollen-tubes and embryo-sacs possessing the same dominant *S* allele, for which specific incompatibility substances are present and compatible reaction between the pollen-tubes and embryo-sacs either of which have the recessive *S* allele and therefore lack the specific incompatibility substances. Thus, in incompatible pollinations normal fertilization occurs in embryo-sacs containing the recessive *S* allele but the growth of the embryo is later stopped, presumably by the diffusion of toxic substances from the incompatible embryo-sacs of the same ovary. These processes are controlled by the *S* gene complex alone. Nevertheless, to explain the self-incompatibility of the hybrid between members of two different self-compatible populations, Cope found it necessary to postulate two independent loci; this seems unwarranted.

Three kinds of *S* allele interactions are known in a diploid cell: (1) *dominance* of one allele over the other; (2) *independent* action of both alleles and (3) interaction leading to *competition* between the alleles neither of which are able to express themselves fully.

The first two interactions have already been demonstrated in *T. cacao*. It is suggested here that competitive interaction, would explain the self-compatibility of certain populations and the self-incompatibility of the hybrids between members of two such different populations. A tree having two competitively interacting *S* alleles would not produce enough specific pollen growth substance for either of the two alleles and would thus be fully self-compatible. A cross between two such unrelated trees would produce progeny all of which may be self-incompatible. Crosses are also possible in which the proportion of self-compatible to self-incompatible plants in the progeny may be 3:1, 1:1 or 1:3. With this hypothesis, the available data are adequately explained on the basis of *S* alleles alone.

The incompatibility system in *T. cacao*, though functionally sporophytic, lies between sporophytic and gametophytic systems and is theoretically still close to the latter, from which it probably originated through interspecific hybridization. This course of evolution is suggested by the results obtained in Oenothera hybrids. Under the influence of some alien cytoplasm, particularly that of a self-compatible species, the activities of certain units of an *S* allele complex, from a gametophytic species, may begin precociously (premeiotically), thereby making the *S* allele action partly sporophytic and partly gametophytic. In *T. cacao* the interaction between *S* allele complexes, which leads to sporophytic reaction, is limited to the units controlling the production of specific growth substances, there being no interaction between the units controlling the production of specific incompatibility precursors, which remain strictly gametophytic in action. This leads to simple gametophytic reaction of the pollen-tubes and embryo-sacs.

In the probable evolution of a fully sporophytic system from a gametophytic one, the competitive interaction between *S* alleles, which bring about self-compatibility, must have been eliminated by selection (Pandey, 1960). The occurrence of fully operative *S* alleles, showing competitive interaction in self-compatible populations of *T. cacao*, indicates that the present system of self-incompatibility is of comparatively recent origin.

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## OBSERVATIONS ON THE SEXUAL BEHAVIOR OF *DROSOPHILA EQUINOXIALIS* AND *DROSOPHILA PROSALTANS*

Several works (Hoenigsberg and Koref Santibanez, 1959a, b, c) have shown that incipient sexual isolation can be observed at the sensorial level. Some aspects of courtship discrimination may result in preferences which in the end may yield observable mating differences (Hoenigsberg and Koref Santibanez, 1959c).

With the male choice method described by others (Dobzhansky and Mayr, 1944; Spieth, 1949) and by the method of employing silver paint on the thorax to distinguish the females more readily (Knight, Robertson and Waddington, 1956), the authors studied the possible sensorial discrimination of *D. equinoxialis* and *D. prosaltans*—both from the American tropics.

*D. equinoxialis* were obtained from seven natural populations, representing four geographical areas:

(1) Cuba: one strain from Cienfuegos (C 25) and one from Baracoa (C 101).
(2) Costa Rica: both strains came from Santa Ana but were caught at different times (CR 9 A and CR 9 B).
(3) Colombia: one strain from Santa Marta (Co 26 F) and one from Bucaramanga (Co 26 G).
(4) Brazil: only one strain was used (Br). It came from Tefe, Amazonas.

The specimens of *D. prosaltans* originated in three areas:

(1) Mexico: one strain from Huichenhugan (Me).
(2) Trinidad: one strain from Sangre Grande (Tr).
(3) Brazil: one strain from Cantareira, Sao Paolo (SP).

All flies were kept at constant temperature (23°C) on laboratory corn meal and sugar medium. Within a few hours from hatching males and females were separated, to be used in the observations at four days of age. Observations were 30 minutes long as a maximum, but were terminated the moment copulation took place. This note refers to the information gathered from 662 *D. equinoxialis* males and 153 *D. prosaltans* males observed in courtship. Insemination analysis was done on 16 females from two different localities which had been placed in a food vial with eight males from one of these localities. As in courtship analysis, the females of one of the two localities were marked with paint on the thorax to facilitate recognition. Ample time (12 hours) was allowed to permit recovery from etherization. After a period of three hours for *D. equinoxialis* and two hours for *D. prosaltans* (23°C) the females were removed from their food vials and were dissected, using a binocular microscope to examine the ventral receptacle and spermathecae for the presence of sperm. For the insemination study alone 2524 *D. equinoxialis* and 780 *D. prosaltans* females were dissected.

Since a detailed analysis of all the data is not possible in this communication, it is sufficient to mention in passing the general preferential courtship which Colombian males have for the females of their own locality. The same tendency, but with a greater number of examples, was found in the

male Brazilian representatives of *D. equinoxialis*. Courtship is considered as having 15 features which are repeated constantly during the ritual (Spieth, 1952). These elements of courtship are: (1) orientations of the male toward the females, (2) tapping the female with fore tarsi, (3) wing vibration, (4) lickings, (5) kicking by the female, (6) fluttering, (7) circling, (8) acceptance response by opening wings, (9) mounting trial, (10) decamping, (11) wing closure, (12) wing opening as a sign of encouragement, (13) running as a repulsion sign, (14) standing of the female while the male courts her, (15) total duration of the courtship.

TABLE 1

Number of orientations and tappings by *D. equinoxialis* males from Brazil. The + sign indicates preference towards own female; the − sign indicates a preference toward the other female, while ind. refers to males whose direction of courtship was random.

| ♂ | ♀♀ | Orientations | | | | | | Tappings | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Own | Other | | | | | Own | Other | | | |
| | | n | % | % | $\chi^2$ | P% | | n | % | % | $\chi^2$ | P% | |
| Br | Br, C 25 | 39 | 82.0 | 18.0 | 8.01 | 1 | + | 142 | 71.8 | 28.2 | 13.5 | 1 | + |
| Br | Br, C 101 | 34 | 79.4 | 20.6 | 5.38 | 1 | + | 137 | 96.3 | 3.7 | 58.8 | 1 | + |
| Br | Br, CR 9 A | 52 | 57.6 | 42.4 | 0.61 | 30–50 | ind. | 167 | 55.1 | 44.9 | 0.8 | 30–50 | ind. |
| Br | Br, CR 9 B | 25 | 96.0 | 4.0 | 10.5 | 1 | + | 146 | 96.5 | 3.5 | 63.3 | 1 | + |
| Br | Br, Co 26 F | 15 | 86.6 | 13.4 | 4.00 | 2–5 | + | 38 | 100.0 | 0.0 | 18.0 | 1 | + |
| Br | Br, Co 26 G | 61 | 80.3 | 19.7 | 11.2 | 1 | + | 211 | 88.1 | 11.9 | 61.4 | 1 | + |

In table 1 we show the differential display of males from Brazil, manifested in distant stimuli (orientations), and in proximal stimuli (tappings) directed toward females from either of two localities. A tendency toward preference of their own kind is consistent in wing vibrations, circlings, courtship time, copulations, lickings and standing position of the females. Only Colombian males of *D. equinoxialis* showed discrimination in courtship, showing preferences not only in proximal stimuli, but in the features of courtship which convey distant stimulation (orientations, wing vibrations, etc.). All the other strains of *D. equinoxialis* showed neither distant nor proximal preferences in courtship with the females from their own choice.

The data obtained by observation of *D. prosaltans* males indicate a clear case of courtship and copulation preference for females from their own locality.

However, there were distinct cases of random choice when the female from outside the male's locality was from Sao Paolo. Orientations, as can be seen in table 2, were less used in discrimination than were the other elements of courtship.

The authors conclude that sensory discriminations which yield preferential courtship and copulations in natural populations do exist and that they favor immiscibility. In nature, where these mechanisms are presumably effective in marginal populations involving contiguous geographical strains,

TABLE 2

Number of orientations and tappings done by *D. prosaltans* from Trinidad, Mexico, and Sao Paolo.

| ♂ | ♀♀ | Orientations | | | | | | Tappings | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Own | Other | | | | | Own | Other | | | |
| | | n | % | % | $\chi^2$ | P% | | n | % | % | $\chi^2$ | P% | |
| Tr | Tr, Me | 136 | 63.2 | 36.8 | 4.76 | 2-5 | + | 306 | 68.3 | 31.7 | 28.4 | 1 | + |
| Tr | Tr, SP | 141 | 67.3 | 32.7 | 8.51 | 1 | + | 310 | 59.6 | 40.4 | 5.31 | 1-2 | + |
| Me | Me, Tr | 37 | 78.3 | 21.7 | 5.95 | 1-2 | + | 158 | 93.0 | 7.0 | 60.8 | 1 | + |
| Me | Me, SP | 92 | 55.4 | 44.6 | 0.54 | 30-50 | ind. | 475 | 77.0 | 23.0 | 69.5 | 1 | + |
| SP | SP, Me | 55 | 54.5 | 45.5 | 0.22 | 50-70 | ind. | 175 | 69.1 | 30.9 | 12.8 | 1 | + |
| SP | SP, Tr | 199 | 48.7 | 51.3 | 0.06 | 80 | ind. | 237 | 63.4 | 36.6 | 10.3 | 1 | + |

natural selection may act to develop further the mechanisms of self recognition.

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